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TAF 15, a possible target for gene regulation by nuclear receptors

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Abstract

This thesis is based on the protein TAF 15. As a TATA binding protein associated factor it is part of the basal transcription factor TFIID, but also exhibits a variety of other functions as a member of the unique TET family.

I found TAF 15 in a Tandem Affinity Purification Assay as an interaction partner for ISG 12. ISG 12 is a small interferon stimulated gene of approximately 12kDa localized in the nuclear membrane. It is speculated to be an inducible part of the nuclear pore complex. It is highly upregulated in atherosclerotic lesions and acts proinflammatory by decreasing the synthesis of antiinflammatory factors. This effect is achieved by an ISG 12 dependent export of nuclear receptors from the nucleus to the cytoplasm.

I investigated complex formations between ISG 12, TAF 15 and nuclear receptors, especially NR4A1. TAF 15 was found to interact with ISG 12 and several nuclear receptors including NR4A1, PPARα and RXRα as well as the transcription factor p65. All of these factors were previously identified to interact with ISG 12. Additionally, ISG 12 was able to downregulate the transcriptional activity of all these factors significantly.

Silencing of TAF 15 by siRNA ultimately led to a decreased complex formation between ISG 12 and the tested proteins. Furthermore, silencing of TAF 15 abolished the export of NR4A1 to the cytoplasm, even when ISG 12 was overexpressed. Surprisingly, a knock out of TAF 15 led to a stronger signal in a fluorescence resonance energy transfer experiment, suggesting that TAF 15 is not actually necessary for ISG 12 to form complexes with nuclear receptors, but rather stabilizes them.

Zusammenfassung

Diese Diplomarbeit beschäfftigt sich mit dem Protein TAF 15. Es gehört zu den TATA binding associated factors und ist somit ein Teil des Transkriptionsfaktors TFIID. Darüberhinaus erfüllt es noch eine Vielzahl anderer Funktionen als Mitglied der einzigartigen TET Familie.

TAF 15 wurde in einem Tandem Affinity Purification Assay gefunden, als ein Interaktionspartner von ISG 12. Bei ISG 12 handelt es sich um ein kleines Interferon induziertes Gen. Das Protein hat eine Größe von ca. 12kDa und befindet sich in der Kernmembran. Weiters wird spekuliert, dass ISG 12 ein induzierbarer Teil der Kernpore sein könnte. In atherosklerotischen Lesionen wurde ISG 12 in überexpremierten Mengen gefunden, wo es proinflammatorisch wirkt indem es die Produktion von antiinflammatorischen Faktoren vermindert. Die Ursache für diesen Effekt liegt in einem ISG 12 abhängigen Export von nuklearen Rezeptoren aus dem Kern ins Zytoplasma.

Aufgrund dessen untersuchte ich die Komplexbildung zwischen ISG 12, TAF 15 und nuklearen Rezeptoren, wobei mein Hauptaugenmerk NR4A1 galt. Die Ergebnisse zeigten eine Interaktion zwischen TAF 15 und ISG 12, sowie mit den nuklearen Rezeptoren NR4A1, PPARα und RXRα. Desweiteren wurde der Transkriptionsfaktor p65 als Interaktionspartner von TAF 15 identifiziert. Diese Proteine wurden in früheren Experimenten bereits als Interaktionspartner von ISG 12 bestättigt. Zusätzlich hatte ISG 12 den gleichen negativ regulierenden Effekt auf die Transkriptionsaktivität dieser Faktoren.

Die Herunterregulierung von TAF 15 mittels siRNA führte zu einer verminderten Komplexbildung zwischen ISG 12 und den vorher genannten Proteinen. Weiters verhinderte die Herunterregulierung von TAF 15 den iSG 12 abhängigen Export von NR4A1 ins Zytoplasma. In einem fluorescence resonance energy transfer Experiment führte es überraschenderweise zu einer verstärkten Interaktion zwischen ISG 12 und NR4A1. Dies weist darauf hin, dass TAF 15 für die Interaktion möglicherweise nicht notwendig ist, es aber bereits entstandene Komplexe stabilisieren könnte.

1. Introduction

1.1 Atherosclerosis

Atherosclerosis is a cardiovascular disease, which is nowadays the most common cause of death in the developed world [1]. It is characterized by the World Health Organization as *“a variable combination of changes in the intima of arteries consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes”.*

The continuous built-up of fatty materials on the inside of artery walls results in a chronic inflammatory response, leading to the accumulation of macrophages and the formation of plaques.

Atherosclerosis starts already in early adolescence [2], but is asymptomatic in the beginning. Its progression is discontinuous and can stagnate at times, which is on the other hand accompanied by periods of rapid growth. The acceleration of plague formation is affected by obesity, gender, hypertension, hyperlipidemia, excessive uptake of saturated fats, diabetes mellitus, lack of physical exercise and genetic factors. In adulthood atherosclerosis is usually wide spread throughout the arterial system. Plaque formation results in a reduction of lumen diameter, thus leading to higher speeds of blood flow. This eventually leads to plaque rupture and clots inside the artery lumen over the ruptures. These clots mostly heal and form arterial stenoses which can result in stable syndromes like insufficient blood supply to tissue. The development of stenoses is chronic and progresses slowly, whereas the formation of a thrombus is an acute event. It is also caused by plague rupture and slows down blood flow rapidly, which can ultimately lead to myocardial infarction or stroke.

Narrowing of the artery lumen also leads to artery enlargement, which can compensate for the reduction of lumen [3]. The recurring hypoxic stimuli can additionally induce the formation of collateral vessels, again counteracting the effects of vessel narrowing. However, if the artery enlargement becomes excessive, it can result in an aneurysm [4], which is a blood-filled dilation of a blood vessel caused by weakening of the vessel wall.

1.2 Atherosclerotic development and progression

The composition of a normal blood vessel is made up of three distinct layers, separated by an internal and external tissue. Arteries and veins are similar in their design, but the three layers are better distinguishable in arteries.

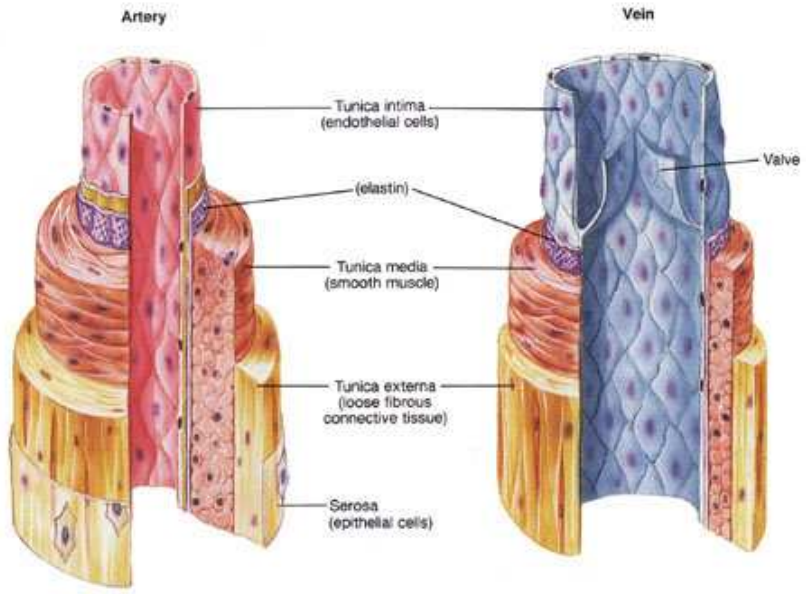

Figure 1.1: Schematic drawing of the different layers in veins and arteries [78].

The tunica intima, a thin endothelial monolayer, is the innermost layer of the blood vessel and has direct contact with the blood stream. It is structurally supported by the internal elastic lamina.

The tunica media, a layer of smooth muscle cells, is responsible for maintaining the vascular tone. This layer is more developed in arteries than in veins.

The tunica adventitia is seperated from the tunica media by the external elastic lamina and consists of fibroblasts, collagen bundles and proteoglycans. It is the outermost layer of the blood vessel [5].

The vascular endothelium is responsible for regulating many important functions, including vascular tone, maintaining blood circulation, coagulation and fluidity. It is also involved in regulating inflammatory responses [16]. In a healthy vessel, white blood cells do not bind to the endothelium, it even acts against an adherence by secreting anti-coagulation mediators.

But gene expression in endothelial cells can be altered by certain cardiovascular risk factors, such as hypertension, hyperglycaemia, hypertriglyceridemia and hypercholesterolemia. These risk factors lead to the expression of adhesion molecules on the endothelial surface. The most important adhesion molecules are the vascular cell adhesion molecule-1 (VCAM-1) and the inter-cell adhesion molecule-1 (ICAM-1) [11].

To trigger an inflammatory response lipoproteins, like low-density lipoprotein (LDL), have to be become oxidized. They accumulate in the intima of an artery where they bind to components of the extracellular matrix [9]. If the LDL concentration is high in the blood stream, more of it is bound and the chance of oxidation increases [17], leading to a variety of biologically active products, like lipid hydroperoxides, lysophospholipids and carbonyl compounds.

These particles make up the so-called “fatty streaks”, the first step in atherosclerotic development [6]. These “fatty streaks” are often removed by an acute inflammatory response, but in some cases the acute response turns into a chronic inflammatory response. In this case the persistence of chronic inflammation is responsible for the progression of the disease, even though the triggering event is already terminated [7].

A result of the ongoing inflammatory response is the activation of transcription factors like NfkB and AP1 [8]. These transcription factors are now responsible for the expression of adhesion molecules like VCAM-1 and ICAM-1, as mentioned above. They are located at the endothelial surface and contact leukocytes. Other surface molecules including P-selectin, E-selectin, L-selectin and various integrins enable leukocytes to traverse the endothelial layer by diapedesis between cellular junctions.

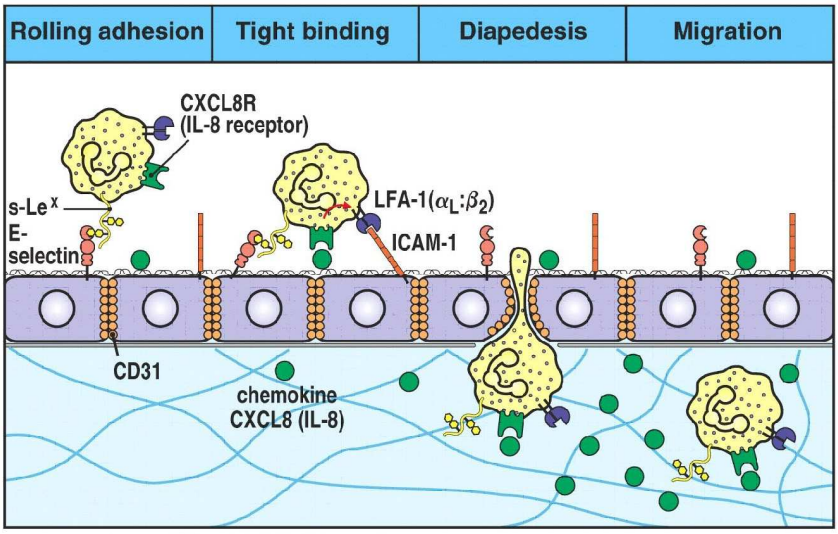

Figure 1.2: Different stages in leukocyte rolling and diapedesis.

First leukocytes are slowed down by weakly binding to selectins, which results in a rolling adhesion. That is further improved by integrin binding and ICAM-1 binding. This directed migration of leukocytes is regulated by a chemoattractant gradient in the atheroma [10].

Monocytes that reach the intima differentiate to macrophages due to the macrophage colony stimulating factor (MCSF) that is produced there.

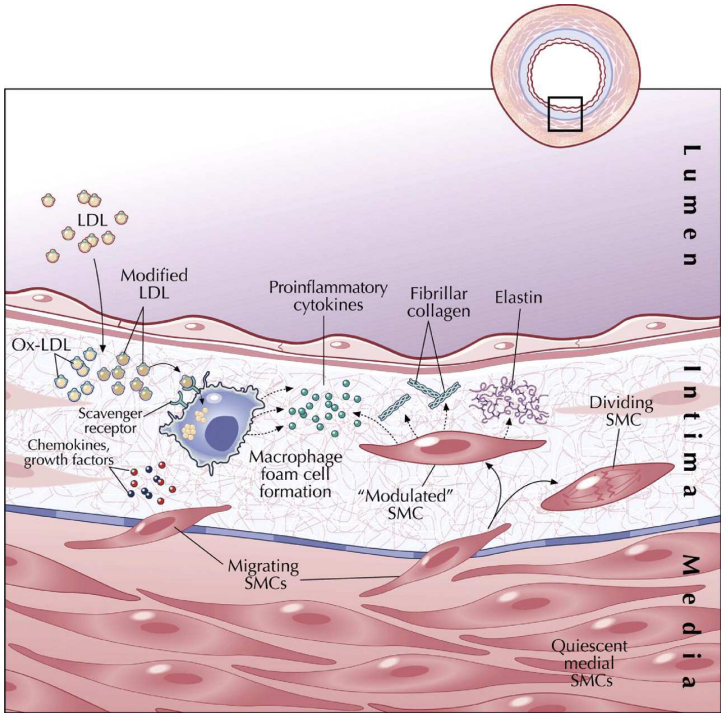

Figure 1.3: Early stages of plague formation in the tunica intima. Differentiation of monocytes to macrophages and accumulation of modified LDL in foam cellls [27].

Macrophages increasingly express scavenger receptors, like scavenger receptor A (SRA) or CD36 [13]. The expression of these receptors on the surface of macrophages results in the internalization of different forms of modified lipoproteins, like oxidized LDL. Lipoproteins are stored in cytoplasmic droplets, which lets macrophages swell as more and more lipoproteins are internalized. This leads to the formation of foam cells [14], which in turn secrete growth factors and cytokines. So the initial beneficial function of macrophages to clear the intima from modified lipoproteins is itself the next step towards plague formation. Growth factors and cytokines released from foam cells themselves act in a pro-inflammatory manner, increasing the recruitment of monocytes, macrophages, T lymphocytes and mast cells. Additionally they activate the classical and alternative complement pathway, stimulating the proliferation and migration of smooth muscle cells from the tunica media. These SMC form a fibrous capsule covering the “fatty streak”. Apoptosis of foam cells leads to the release of previously incorporated lipoproteins, which accumulate together with cell debris in a lipid rich centre of the plague.

Adding to the ongoing plague formation are T lymphocytes, which also migrate into the intima. They are mostly Th1 cells and they contribute by releasing IL-2, TNFα, TNFβ and IFNγ. These cytokines stimulate pro-inflammatory cells already present in the atheroma. Furthermore they enhance proliferation of smooth muscle cells. Th2 cells, which in general exhibit anti-inflammatory effects, play a minor role due to their lack of numbers [15].

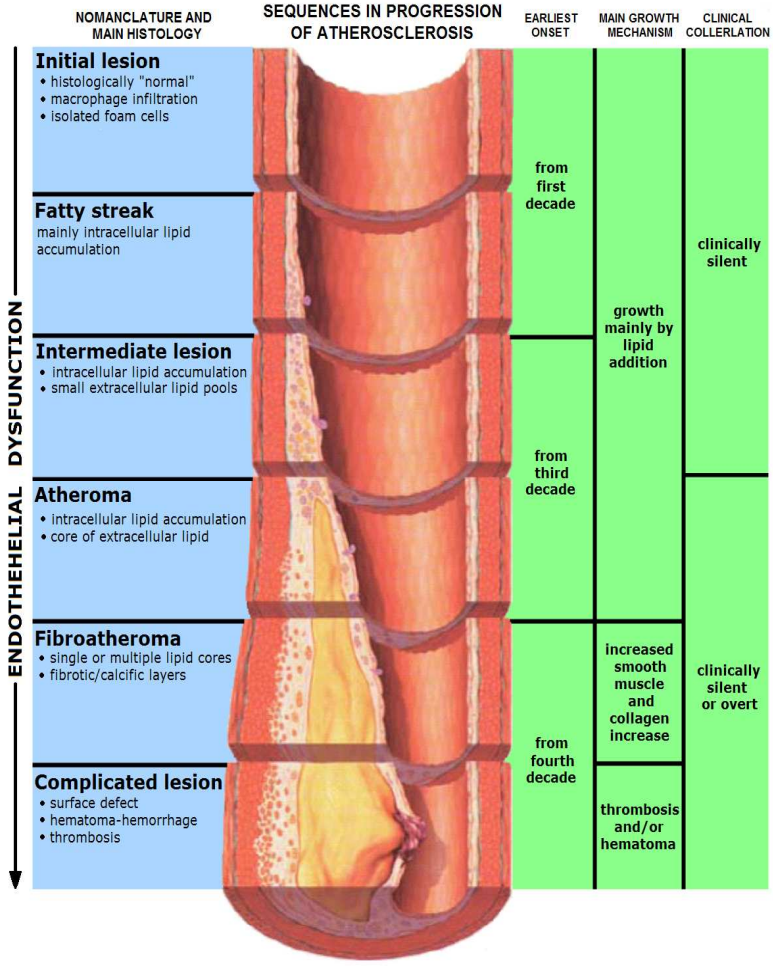

Figure 1.4: Distinct stages in the development of complicated lesions and corresponding time of onset.

The accumulation of smooth muscle cells leads to the development of more complicated atheroma, which growing size significantly decreases the lumen of the vessel. First clinical manifestations arise when the blood flow gets disturbed.

The fibrous capsule covering the plague is continuously degraded by collagenases that are produced by macrophages. Collagenase also inhibits the new synthesis of collagen by smooth muscle cells. As a result the capsule becomes thinner and the risk of rupture increases. Once the plague breaks open tissue factors of macrophages are released into the blood stream where they activate platelets, which in turn activate the system of coagulation which results in thrombus formation [12]. A thrombus is a complex structure of leukocytes, erythrocytes, platelets and fibrin as the final step of hemostasis. It can lead to myocardial infarction, stroke or critical limb ischemia. In the case of a nonlethal outcome, the following wound healing response again stimulates the proliferation of smooth muscle cells, further

narrowing the lumen of the blood vessel.

Increased amounts of lipids in the blood are the starting point for atherosclerosis, but the critical step in its formation is the switch from an acute immune response to a chronic one. So in fact, the initial inflammatory response to clear oxidized LDL from the intima eventually leads to plague formation and the onset of disease.

1.3 Nuclear Receptors

Nuclear receptors (NRs) are proteins found in animals where they act as transcription factors [19] in regulating gene expression. They are involved in the control of development, homeostasis, metabolism and reproduction. NRs bind to lipophilic molecule within the cell and in response work together with other proteins to induce a downstream effect in either up or down regulating the expression of specific genes.

NRs themselves have the ability to directly bind to DNA, but first they need to be activated by a ligand. Ligand binding results in a conformational change in the receptor and a loss of corepressors. With the dissociation of corepressors NRs bind to coactivators which in turn can interact with the transcriptional machinery.

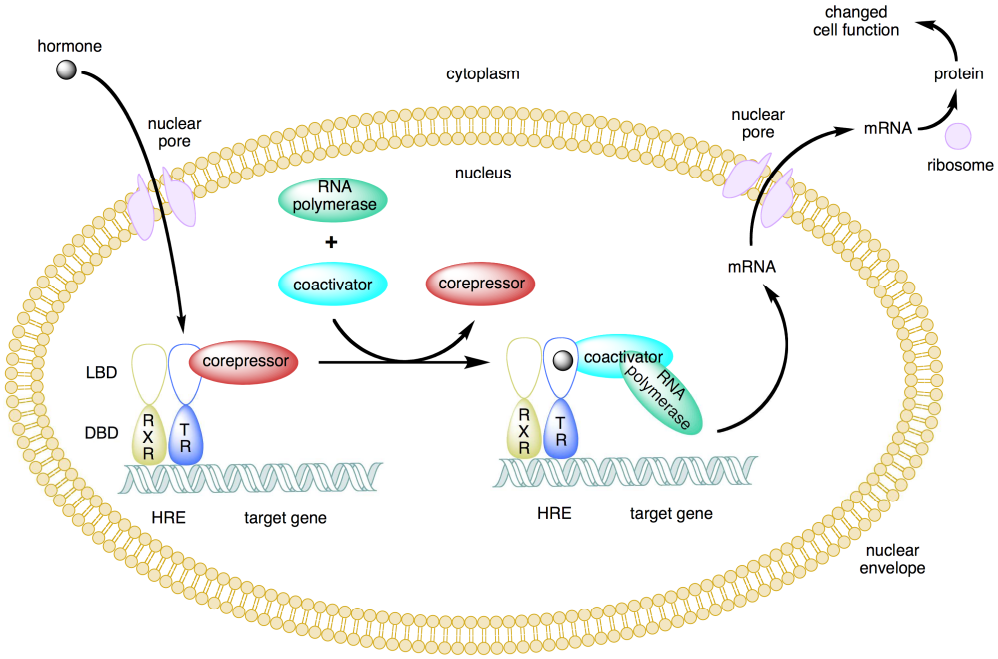

Figure 1.5: Mechanism of transcriptional activation by the thyroid hormone receptor.

All NRs consist of five domains. The N-terminal domain includes the activation function 1 (AF1) which is ligand independent [20]. It is involved in transactivation by interacting with co-regulators. The N-terminal regulatory domain is highly variable in sequence between different NRs. Adjacent to the N-terminus is the DNA binding domain (DBD), which is a highly conserved region. It is made up of two zinc fingers

which can bind to specific sequences of DNA called hormone response elements (HRE). The most commonly recognized sequence is 5'-PuGGTCA-3'. HRE sequences can consist of merely one such sequence, or of two copies as a palindrome or a direct repeat [18]. The DBD is linked to the ligand binding domain (LBD) via a flexible hinge region. The LBD is a moderately conserved region at the C-terminus of NRs. The LBD structure is a so-called alpha helical sandwich fold consisting of 8 alpha helices that make up the ligand binding cavity. In addition the LBD contributes to receptor dimerization. Finally the LBD contains the activation function 2 (AF2) at the C-terminal end of the receptor [20]. The function of AF2 is ligand-dependent contrary to AF1.

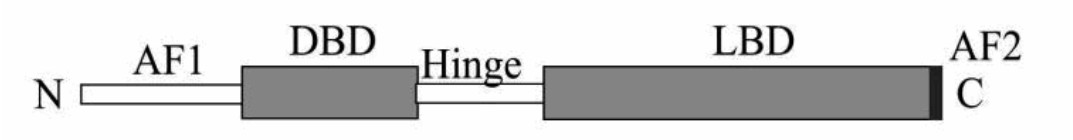

Figure 1.6: Different structural domains common in nuclear receptors.

NRs are divided in six subfamilies according to sequence homology [18]:

Subfamily 1: Thyroid Hormone Receptor-like

Is the largest subfamily and contains Thyroid hormone receptors (TR), Retinoic acid receptors (RAR), Peroxisome proliferator-activated receptors (PPAR), Rev-ErbA, RAR-related orphan receptors (ROR), Liver X receptor-likes (LXR) and Vitamin D receptor-likes.

Subfamily 2: Retinoid X Receptor-like

Contains Hepatocyte nuclear factor-4 (HNF4), Retinoid X receptors (RXR), Testicular receptors (TR), TLX/PNR and COUP/EAR.

Subfamily 3: Estrogen Receptor-like

Also called steroid or sex hormone receptors, contains Estrogen receptors (ER), Estrogen related receptors (ERR) and 3-Ketosteroid receptors (glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor and androgen receptor).

Subfamily 4: Nerve Growth Factor IB-like

Contains Nerve Growth factor IB (NR4A1 or Nur77), Nuclear receptor related

1 (NURR1) and Neuron-derived orphan receptor 1 (NOR1).

Subfamily 5: Steroidogenic Factor-like

Contains Steroidogenic factor 1 (SF1) and Liver receptor homolog-1 (LRH-1).

Subfamily 6: Germ Cell Nuclear Factor-like

Contains only Germ cell nuclear factor (GCNF).

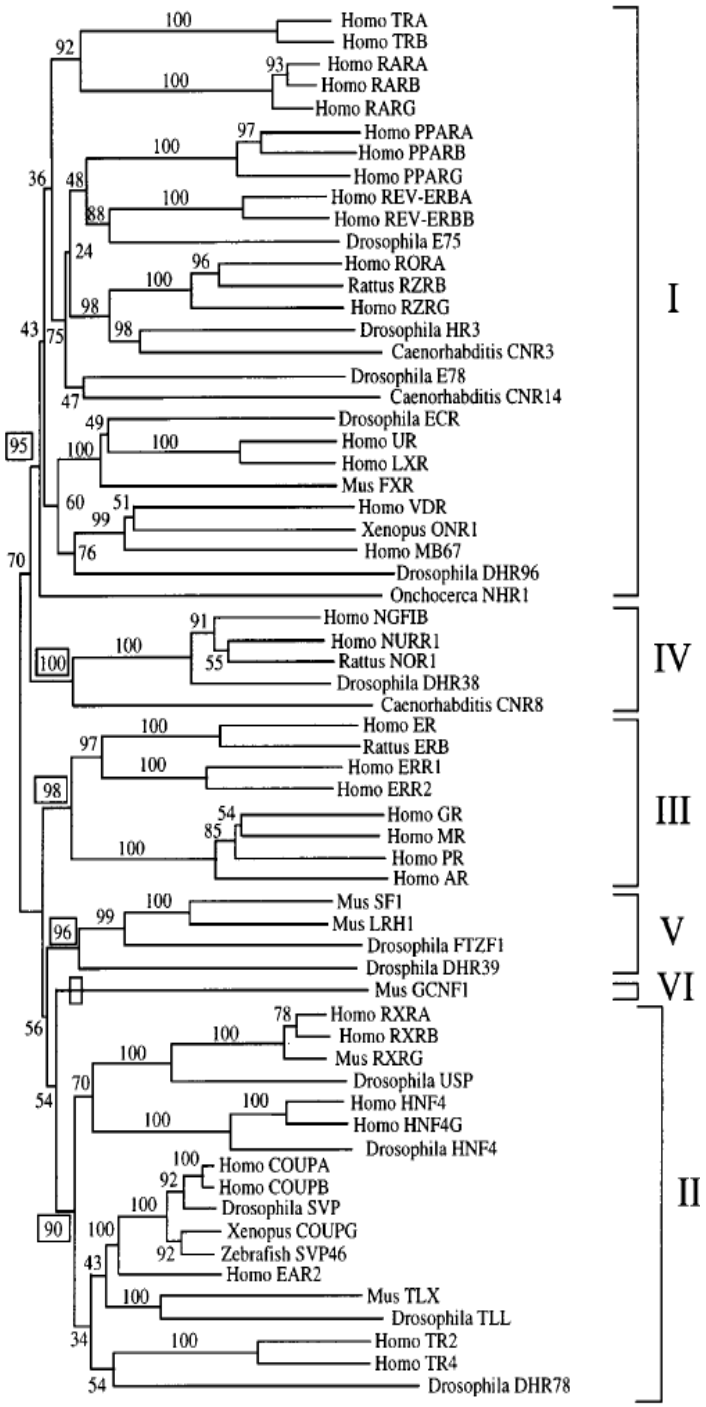

Figure 1.7: Phylogenetic tree of nuclear receptors. The subfamilies are indicated in brackets. The numbers display the bootstrap confidence in percentage [18].

1.3.1 The NR4A Subfamily

The NR4A Subfamily of nuclear receptors consists of three members, Nur77 also called NR4A1, TR3, NGFI-B or Nak1; Nurr1 also known as NR4A2 or NOT; and NOR-1 or indicated as NR4A3 or MINOR. They all comprise the same structural features as other NRs, which are mentioned above, but so far, no ligands have been identified. Therefore they are classified as orphan receptors. Crystallography of the ligand binding domain of Nurr1 revealed that the ligand binding pocket is filled with hydrophobic amino acid side chains [21]. This could mean that Nurr1 is completely unable to interact with ligands, but a possible induced fit of small unknown ligands cannot be excluded (for example C-DIMs or cytosporine). Due to a high homology in the LBDs of all three members a similar function is proposed for the LBDs of Nur77 and NOR-1. The DNA binding domain shows the highest homology of amino acid sequence with about 90%. This suggests an evolutionary relationship for the NR4A members and that they might descend form one ancestral gene.

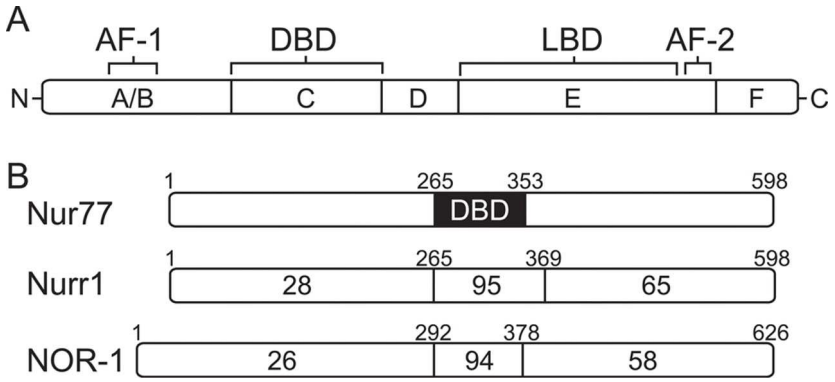

Figure 1.8: (a) Structural domains of NRs. (b) Amino acid sequence alignment of NR4A family members. Numbers give similarity in percent compared to Nur77.

NR4A NRs bind with their DNA binding domain as monomers to the NGFI-B response element (NBRE, AAAGGTCA). They can also bind as homodimers to the Nurr1 response element (NurRE, TGATATTTn6AAATGCCA) which is an inverted repeat containing two NBRE related sequences separated by 6 nucleotides [22]. Besides, Nur77 and Nurr1, but not NOR-1, can heterodimerize with the retinoid X receptor (RXR, a member of NR Subfamily 2) and bind to the DR5 response element (AGGTTCACCGAAAGGTCA) [23].

NR4A nuclear receptors are expressed in many different tissues in the human body and are involved in a variety of processes. Nur77 has been described in cancer cell apoptosis and Nurr1 has an essential role in brain development. They are described as “immediate early genes” because they are transiently and rapidly induced by a number of stimuli. These include inflammatory cytokines, growth factors, fatty acids, oxidized lipids and lipopolysaccharides (LPS) as well as physical stimuli. Transcription factors involved in activating NR4A NRs gene expression are cAMP response element binding protein (CREB), activator protein-1 (AP-1), myocyte enhancing factor-2 (MEF-2), nuclear factor of activated T-cells (NFAT) and NFκB [24] [25] [74]. In addition to gene expression also posttranslational modifications affect the transcriptional activity of NR4A NRs. NR4A receptors can be phosphorylated at either serines or threonines by the kinase Akt, the extracellular signal-regulated kinase 2 (ERK2) or the Jun N-terminal kinase. The phosphorylation of Nur77, for example, at serine-350 by Akt inhibits its DNA binding capacity, which leads to a down regulation of Nur77s transcriptional activity [26].

In recent years, more and more publications have been published about the expression of all three NR4A members in human atherosclerotic lesions, fasted liver, skeletal muscle and adipose tissue [44].

1.3.2 NR4A nuclear receptors in metabolic processes

A dysregulated lipid and glucose mechanism are major risk factors for the development of vascular disease [28]. It has been shown that all three members of the NR4A Subfamily are induced in cultured mouse hepatocytes by glucagon [29]. This induction is mediated by CREB, a transcription factor for hepatic gluconeogenesis. After induction, Nur77 itself induces the expression of glucose-6-phosphatase (G6pc) and fructose biphosphatase 1 (Fbp1), two gluconeogenic enzymes. Furthermore a couple of other proteins involved in glucose metabolism are also induced, like Fbp2, glucose transporter Glut2(Slc2a2) and enolase 3 (Eno3). Additionally, overexpression of Nur77 in mouse liver resulted in an increase of gluconeogenesis and an increase of blood glucose after fasting [29].

Lipids and glucose are utilized in skeletal muscle cells for the generation of energy. The activation of adrenergic receptors by β-adrenergic receptor agonists induces

lipolysis in skeletal muscle cells. These agonists also induce increased expression of Nur77 and NOR-1 protein. When Nur77 is knocked down with siRNA it results in down regulation of several genes involved in lipolysis and energy expenditure, mainly glucose transporter 4 (Glut4), caveolin 3 (CAV3), CD36 and uncoupling protein 2 (UCP2). According to this, lipolysis is decreased in skeletal muscle cells upon inactivation of Nur77. NOR-1 and Nur77 have also been found to be expressed during myogenesis and knockdown of NOR-1 in skeletal muscle cells increases myostatin expression. So it is believed that NOR-1 is somehow involved in regulating skeletal muscle mass [30] [31].

1.3.3 NR4A nuclear receptors in macrophages

Certain atherosclerotic stimuli, like oxidized LDL, LPS and TNF-α, induce the expression of NR4A nuclear receptors in monocytes and macrophages [32] [33] [34]. Nur77, Nurr1 and NOR-1 are all expressed in atherosclerotic lesion macrophages, especially in areas of plaque activation and remodeling. This indicates an involvement of NR4A family members in the progression of plaque formation [35].

Nur77 works as a pro inflammatory mediator in mouse macrophages by inducing I-κ-B kinase (IKKi) [34]. Induction happens by direct binding of Nur77 to the NBRE sequence in the promoter region of the IKKi gene. On the other hand, it was also shown that an up regulation of NR4A receptors in cultured human THP-1 derived macrophages leads to a reduction in the expression of inflammatory cytokines, like IL-1β, IL-6 and IL-8 [35]. Other proteins downregulated in response to NR4A receptor over-expression are the monocyte chemoattractive protein-1 (MCP-1), the macrophage inflammatory protein (MIP)-1α and MIP-1β. Furthermore an over-expression leads to a decreased uptake of oxidized LDL particles into macrophages, because of a reduced expression of CD36 and scavenger receptor SR-A, thus slowing down the formation of foam cells. Accordingly, a knock down of Nur77 or NOR-1 results in increased inflammatory cytokine production and an enhanced uptake of oxidized LDL. The effect on the inflammatory gene expression is believed to be due to the ability of NR4A NRs to transrepress NFκB. It has been shown that Nur77 down regulates the transcriptional activity of NFκB [36].

1.3.4 NR4A nuclear receptors in VSMC

Vascular smooth muscle cells are normally quiescent contractile cells that are responsible for maintaining vessel wall stability. In the case of local inflammation or vascular damage, they become activated and start to migrate into the intimal compartment of the vessel wall, where they start proliferating.

Expression of NR4A NRs was first observed after the stimulation of SMCs with conditioned media from oxidized LDL activated macrophages. Nur77 was induced in the murine vessel wall only 6 hours after a cuff-induced SMC-rich lesion formation [37] [38]. This is in accordance to the characterization of NR4A NRs as “immediate early genes”. After examining human patients it became evident that NR4A NRs are expressed in activated SMCs in atherosclerotic lesions and other related vascular pathologies like vein-graft disease.

SMCs obtained from NOR-1 deficient mice showed repressed proliferation after activation [39]. Data sustained by a knock down of NOR-1 in SMCs using antisense oligonucleotides showed similar results. Decreased proliferation is accompanied by a decrease in expression of cell cycle proteins cyclin D1 and cyclin D2.

On the other hand, Nur77 shows an inhibitory effect on SMC proliferation, unlike NOR-1. In this case, an over expression of Nur77 in venous and arterial SMCs leads to a reduced proliferation [38]. Mice that express a dominant-negative variant of Nur77 lacking the N-terminal transactivation domain, show enhanced lesion formation upon carotid artery ligation. The dominant negative form of Nur77 blocks the transcriptional activity of all three members of the NR4A subfamily. Reduced proliferation of SMCs is accompanied by an up regulation of the cell cycle inhibitor $p27^{Kip1}$ and a decrease in the cell cycle protein cyclin A [37] [38].

Additional experiments revealed a connection between over-expression of Nur77 and the expression of calponin and smooth muscle α actin, suggesting that Nur77 might induce an even more differentiated SMC phenotype [38].

Endogenous NR4A NRs seem to protect against the formation of atherosclerotic plaques, since mice over-expressing Nur77 in the arterial vessel wall show decreased lesion formation and mice over-expressing the dominant negative variant of Nur77 develop larger lesions.

1.3.5 NR4A nuclear receptors in ECs

NR4A NRs are induced in endothelial cells upon stimulation with VEGF, TNF-α or IL-1β [41] [42] [43]. VEGF stimulation also results in a decreased phosphorylation of Nur77 in ECs at the negative regulatory site serine-350 [42].

The only directly regulated gene by Nur77 is the plasminogen activator inhibitor 1 (PAI-1), which is an important inhibitor of vascular fibrinolysis. Nur77 binds directly to an NBRE sequence in the promoter of the PAI-1 gene, inducing PAI-1 expression. Again, cells expressing a dominant negative form of Nur77, show almost no PAI-1 expression even when stimulated with TNF-α [44].

Knock down of NOR-1 in ECs results in decreased proliferation and migration upon VEGF stimulation. Also silencing of Nur77 leads to a decrease in VEGF-induced proliferation [43].

Over-expression of Nur77 accordingly enhanced EC survival and proliferation, together with an induction of cyclin A, cyclin D1, proliferating cell nuclear antigen (PCNA) and transcription factor E2F.

Finally, it was shown that the levels of Nur77 are increased in ECs during angiogenesis. Consistent with those findings, angiogenesis was reduced in Nur77 deficient mice, also reducing tumor cell growth.

1.4 ISG 12

The interferon-stimulated gene 12 (ISG 12) was primarily found to be an estrogen-induced gene, but was later found to be mainly stimulated by type I IFNs [48].

IFNs mediate their biological functions mainly through the activation of ISGs. More than 1000 ISGs have been found till now [45], and their activation starts with the binding of IFN to specific cell surface cytokine receptors. After IFN is bound the receptor chains oligomerize and the nonreceptor tyrosine kinases Janus kinases (Jak 1-3) and tyrosine-specific kinase (Tyk) phosphorylate each other as well as tyrosine residues on the receptor chains. The kinases, once activated, phosphorylate signal transducers and activators of transcription proteins (STATs). The activated STATs move to the nucleus and act as transcription factors [46].

There are two different IFN signaling pathways. Type I IFNs cause STAT1 and STAT2 to heterodimerize and to associate with the DNA-binding protein IFN regulatory factor-9 (IRF-9). Together they form the IFN-stimulated gene factor 3 (ISGF3) complex, which can bind to the IFN-stimulated response element (ISRE) in the promoter region of type I IFN stimulated genes. Type II IFN however leads to a homodimerization of STAT1, forming the IFN-γ activated factor (GAF). GAF binds to IFN-γ activation sites (GAS) in the promoter region of type II IFN stimulated genes [47].

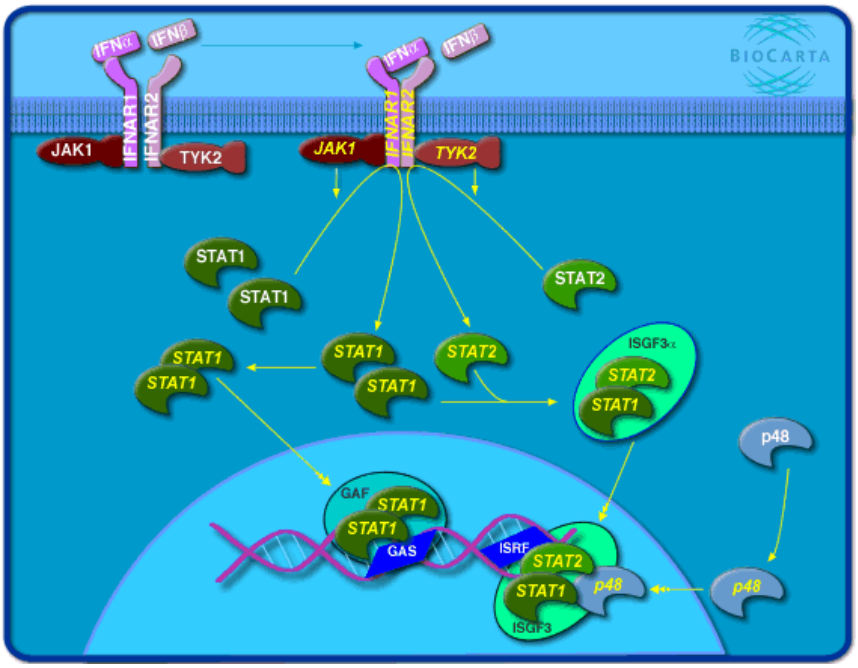

Figure 1.9: Activation of STATs by IFN and the formation of functional transcription factors.

ISG 12 is part of the so called “small ISGs”, it encodes a putative hydrophobic protein with 122 amino acids. Its molecular weight is approximately 12 kDa. As mentioned above it was first found as an estrogen-induced gene in human breast adenocarcinoma cells [48]., however the expression of ISG 12 did not correlate with the presence of estrogen receptors in other cells. Still two estrogen receptor elements were found upstream of the IFN-regulated elements in the ISG 12 promoter region. Further studies revealed that ISG 12 is highly up regulated by type I IFNs [49], especially IFNα, in cells irrespective to their estrogen receptor status, indicating that IFNα inducibility is estrogen independent. This is consistent with the human gene 6-16, which together with ISG 12 is a member of the ISG 12 gene family. In total there are 46 members from 25 organisms belonging to the ISG 12 gene family [50].

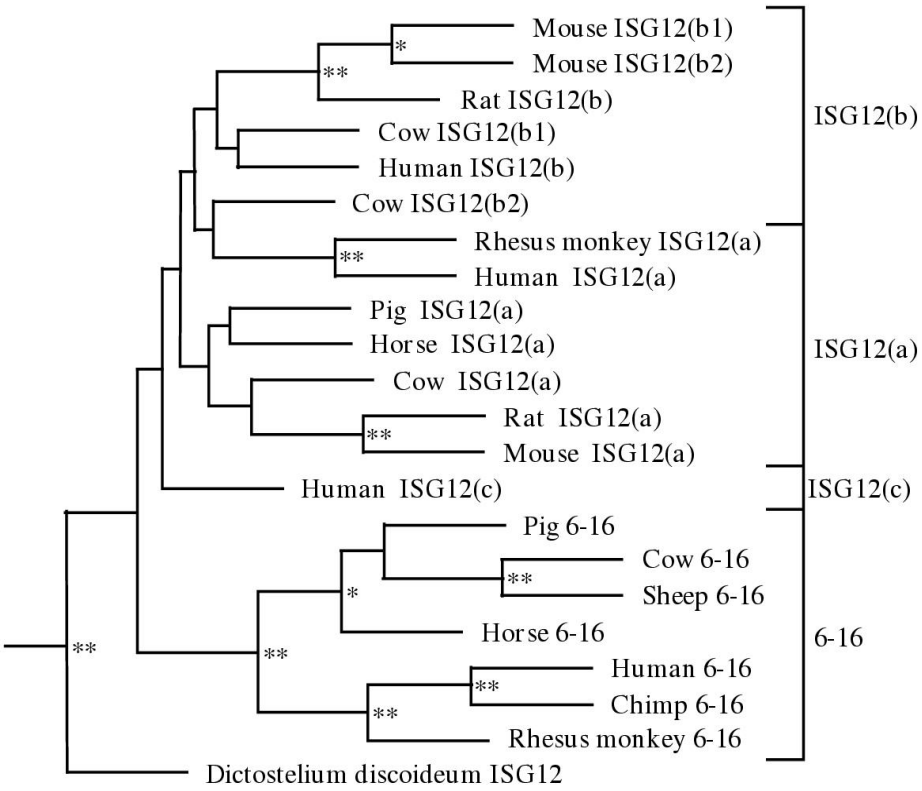

Figure 1.10: Phylogenetic tree of the ISG 12 family. Two stars represent a bootstrap confidence level >85%, one star >60% [50].

The proteins of the ISG 12 family are encoded by the GIP3 and IFI27 genes. The ISG 12 gene is located on chromosome 14q32 with a length of approximately 6kb [48] [51]. Four members of the family were found in human, ISG 12(a), ISG 12(b), ISG 12(c) and 6-16. 6-16 is located on chromosome 1p35. Another three were found in mice coding for 90 amino acid long proteins with a molecular weight of approximately 10 kDa.

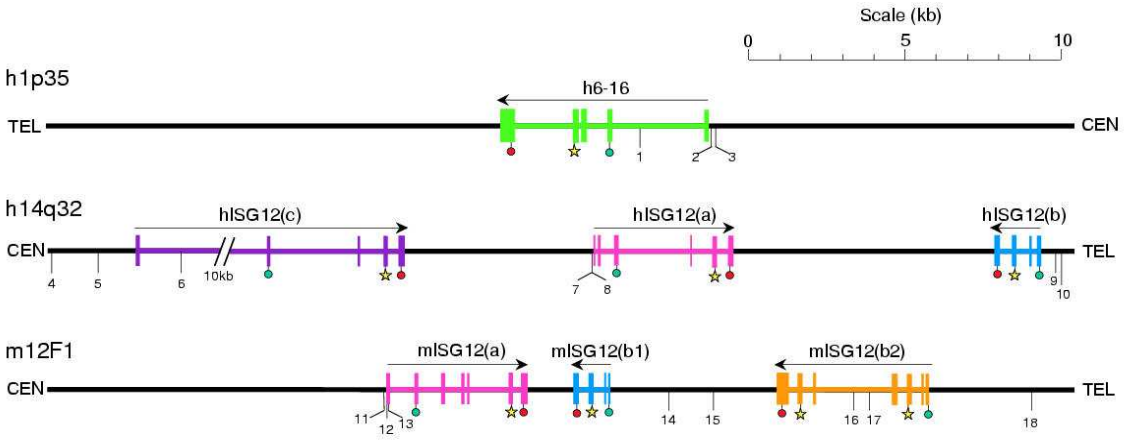

Figure 1.11: Genomic organisation of ISG12 genes in humans and mice. Regions of human chromosomes 1p35 and 14q32, and mouse chromosome 12F1, carrying *ISG12* genes are shown. Transcribed regions are coloured. Translational initiation and termination sites are indicated by green and red circles, respectively. Exons encoding an ISG12 motif are starred.

Human ISG 12 has 5 different splice variants of its mRNA encoding for full length protein [52]. There is a shorter splice variant lacking exon 2, leading to a short isoform of ISG 12, called ISG 12-S [75]. Furthermore there is ISG 12Δ, which has a 9-nt deletion in exon 4. The deletion is in frame and causes a loss of 3 amino acids. The combined isoform ISG 12-SΔ lacks an additional 5 amino acids in its N-terminal region compared to ISG 12-S. Finally the mRNA of ISG 12 has two different 5' UTR splice variants, both encoding a full length ISG 12 protein.

ISG 12 is inserted into the nuclear envelope [52] and it is possible that it is an inducible part of the nuclear pore complex.

It is expressed in a variety of normal tissue like kidney, colon, prostate, placenta, ovaries, testis and endothelium and also in tumors from skin [53] [54], colon, pancreas, kidney, breast and lung.

In recent years, Papac-Milicevic, N. et al. studied the functions of ISG 12 as a modulator in atherosclerosis and restenosis *in vitro* and *in vivo* [56].
ISG 12 was found as an interaction partner of the nuclear receptor NR4A1 in a yeast two hybrid system and was later found to be up regulated 4-times in human atherosclerotic lesions, compared to adjacent non-afflicted areas [56]. Furthermore ISG 12 was expressed upon IFNα stimulation in the monolytic cell line U937, in VSMCs and in human umbilical vein endothelial cells (HUVECs).
First in vitro experiments showed that ISG 12 has the ability to down regulate the transcriptional activity of several nuclear receptors, including NR4A1, NR4A2, PPARα, PPARγ, LXR and FXR. The inability of ISG 12 to affect the transcriptional activity of NR4A3 led to the assumption that ISG 12 is selective for NRs that can form heterodimers with RXR.
The down regulating effect of ISG 12 was found to be due to an export of NRs out of the nucleus. These results were first obtained by over-expressing ISG 12 and EGFP-tagged NR4A1 in HEK cells and were later confirmed in mouse embryonic fibroblasts (MEFs) by increasing the amount of endogenous ISG 12 by IFNα stimulation. Additionally no nuclear export of NR4A1 was seen in ISG 12 deficient MEFs. The same results applied for PPARα [56].
Regarding these results and since ISG 12 is located in the nuclear envelope close to nuclear pore complexes, it is highly likely that ISG 12 binds to NRs and decreases their nuclear localization by influencing their export to the cytoplasm.

ISG 12 deficient mice as well as wild type mice were thereafter subjected to carotid artery ligation and femoral artery cuff experiments [56]. In WT mice, the experiments led to a high up regulation of ISG 12, NR4A1 and monocyte attractant protein 1 (MCP-1), an inflammatory marker gene. On the other hand, knock out mice showed no narrowing of the vessel lumen and no up regulation of MCP-1. This effect possibly involves NR4A1, since NR4A1 and ISG 12 double deficient mice developed restenosis similar to WT mice. Consistent with in vitro data, the localization of NR4A1 in the nucleus was reduced in WT mice in ligated arteries compared to ISG 12 knock out mice.

All this data suggests that the beneficial effect in ISG 12 deficient mice is due to the protective transcriptional activity of nuclear receptors.

1.5 TET Family

TAF 15 together with TLS and EWS are members of the TET family [57]. All members of this unique family are RNA-binding proteins and are structurally and functionally closely related. They share an N-terminal SYGQ-rich region, an RNA-recognition motif, a zinc finger motif and at least one RGG-repeat region [58].

The TET family proteins have all been found in a whole set of cancer associated fusion genes, for example TAF 15 – CIZ/NMP4 [62] from acute leukaemia or TAF 15 – CHN/TEC [63] [64] from extraskeletal myxoid chondrosarcoma. In all cases the N-terminus of either TLS, EWS or TAF 15 acts as a transcriptional activation domain and is fused to an DNA-binding domain of a transcription factor [59] [60] [61]. These newly formed transcription factors are believed to be involved in the formation of a variety of different cancers. In some of these fusions TLS, EWS and TAF 15 can replace each other with out changing the resulting pattern of gene expression, suggesting a functional relationship.

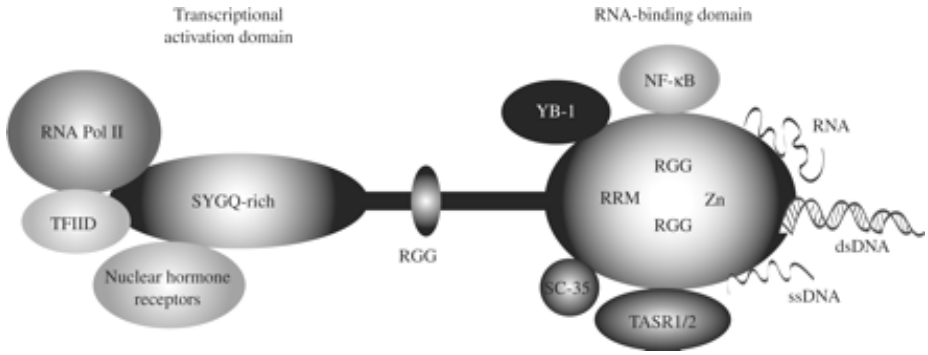

Figure 1.12: An interaction map for TLS [79]

The TET proteins are some of the few proteins found to be able to bind directly to RNA as well as single stranded and double stranded DNA [75] [76] [77]. Given their abilities it is not surprising that they were found to be involved in splicing [65] and RNA shuttling [66] [67]. However these proteins have also been implicated in the process of gene transcription.

TAF 15 is part of the basal transcription factor TFIID and therefore associated with the formation of the pre-initiation complex [68]. TFIID is essential for the initiation of

transcription by RNA polymerase II, by binding to the core promoter and serving as a scaffold for the assembly of the remaining complex. TFIID consists of the TATA-binding protein (TBP) and of several TBP-associated factors, TAFs [69].

TLS and EWS can interact with several gene-specific transcription factors, which probably gives them a more specialized role in regulating gene expression. TLS is additionally an interaction partner for a couple of nuclear hormone receptors like RXR or estrogen receptor [70]. It was also found to interact with the p65 subunit of NFκB, a key regulator of apoptosis [71].

All three members can furthermore interact with transcriptional repressors, like ZFM1 [72], showing that they all are very versatile parts in the initiation or repression of transcription.

2. Objectives

Previous research done by *Nikolina Papac-Milicevic et al* [56] revealed an involvement of ISG 12 in the development of atherosclerosis. ISG 12 was found to downregulate the transcriptional activity of the nuclear receptor NR4A1 almost completely. By cloning different deletion constructs of ISG 12, Sabine Fenzl tried to find the direct interaction site between ISG 12 and NR4A1. Unfortunately no conclusive results could be obtained.

The objective of my work was to identify other interaction partners of ISG 12 and to investigate whether they are somehow involved in the regulation of nuclear receptor activity.

3. Results

3.1 Tandem Affinity Purification Results

To find interaction partners of ISG 12, I tagged myc-ISG 12 with a Tandem Affinity Purification (TAP) [73] tag at the C-Terminus. The resulting C-TAP tagged myc-ISG 12 was stably transfected into HEK293 cells and cultivated. One hundred 175cm^2 flasks were harvested and the cells were lysed. After all the tandem purification steps were completed (see materials & methods) the purified protein complexes were analyzed by mass spectroscopy.

The identified interaction partners of ISG 12 are listed in figure 3.1. Common false positive results are removed.

INTERACTION PARTNER	**LOCALIZATION**	**RELEVANCE**
BASP1	Nucleus and membrane	Associate with WT1 to regulate ist transcription function during development.
FLJ22594	Cytoplasm (cytoskeleton)	Targets myosin phosphatase to the actin cytoskeleton.
CACNA2D1	Membrane	L-type calcium channel, regulates cell metabolism and proliferation.
CD109	Membrane	GPI-glycoprotein, part of TGF-βreceptor system in human keratinocytes, upregulated in tumors.
CDC42BPA	Cytoplasm	Required for cell invasion through the regulation of MLC2 phosphorylation.
CD55	Membrane	GPI-glycoprotein, interruption by CD55 of the complement sequence at an early step in activation halts progression of the cascade and prevents consequent cell injury.

FYN	Membrane	Tyr kinase, required in brain development and mature brain function and regulation of axon growth.
GLG1	Golgi apparatus membrane	GLG1 might have a chaperone function, perhaps playing a role in processing and targeting the growth factor in the cell.
LIMA1	Cytoplasm	Colocalizes with actin stress fibers and focal adhesion plaques.
MARCKSL1	Membrane	May be involved in coupling the PKC and calmodulin systems.
PPP1R12A	Cytoplasm	It is one of the subunits and integral component of the myosin phosphatase.
RAI14	Nucleus and cytoplasm	Highly expressed in several cancer cell lines, precise function remains to be determined.
TAF15	**Nucleus and cytoplasm**	**RNA, dsDNA and ssDNA binding protein that might play specific roles during initiation at distinct promoters**
TLS	**Nucleus and cytoplasm**	**RNA, dsDNA and ssDNA binding protein that might play specific roles during initiation at distinct promoters**

Figure 3.1: List of positive interaction partners of ISG 12 found by a tandem affinity purification and analyzed by mass spectroscopy.

TAF 15 and TLS, both members of the TET family, were the most promising candidates for further investigation, since they were already known to interact with certain nuclear receptors, likewise ISG 12. Additionally, TAF 15 is a part of the basal transcription factor TFIID, which is essential for the initiation of transcription by RNA polymerase II. ISG 12 might in some way interfere with the formation of the pre-initiation complex by binding and retaining TAF 15 and TLS.

3.2 Confirmation of Tandem affinity purification results

To confirm that TAF 15 actually forms a complex with ISG 12, I performed a Co-Immunoprecipitation using an anti-myc antibody to pull down myc-ISG 12. The following Western blot was developed with an anti-TAF 15 antibody. The obtained band from the CoIP had the same size as the band from a cell lysate (control for Input) and was missing in a negative control. For the negative control I used an unspecific rabbit IgG antibody for the pull-down.

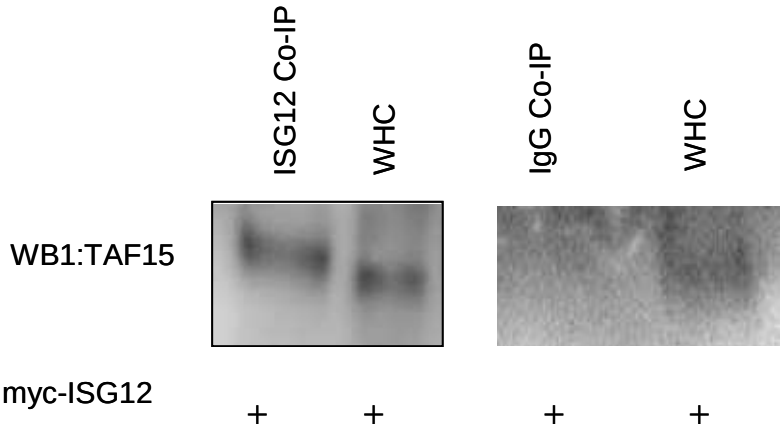

Figure 3.2: Western blot bands from a 10% SDS-Page gel plotted on a PVDF membrane. The membrane was developed with an anti-TAF 15 polyclonal antibody and labelled with a corresponding secondary peroxidase labelled antibody. WHC stands for whole cell lysate.

To further investigate the colocalization between TAF 15 and ISG 12, I transfected HEK293 cells in 2 Well chamberslides with myc-ISG 12. The cells were incubated with primary antibodies against myc and TAF 15 and labelled with fluorescent secondary antibodies. Pictures were taken with a LSM-510 Meta confocal laser microscope.

ISG 12 was labelled green with Alexa Fluor 488 and TAF 15 was labelled red with Alexa Fluor 568. A possible colocalization would lead to yellow fluorescence due to colour interference.

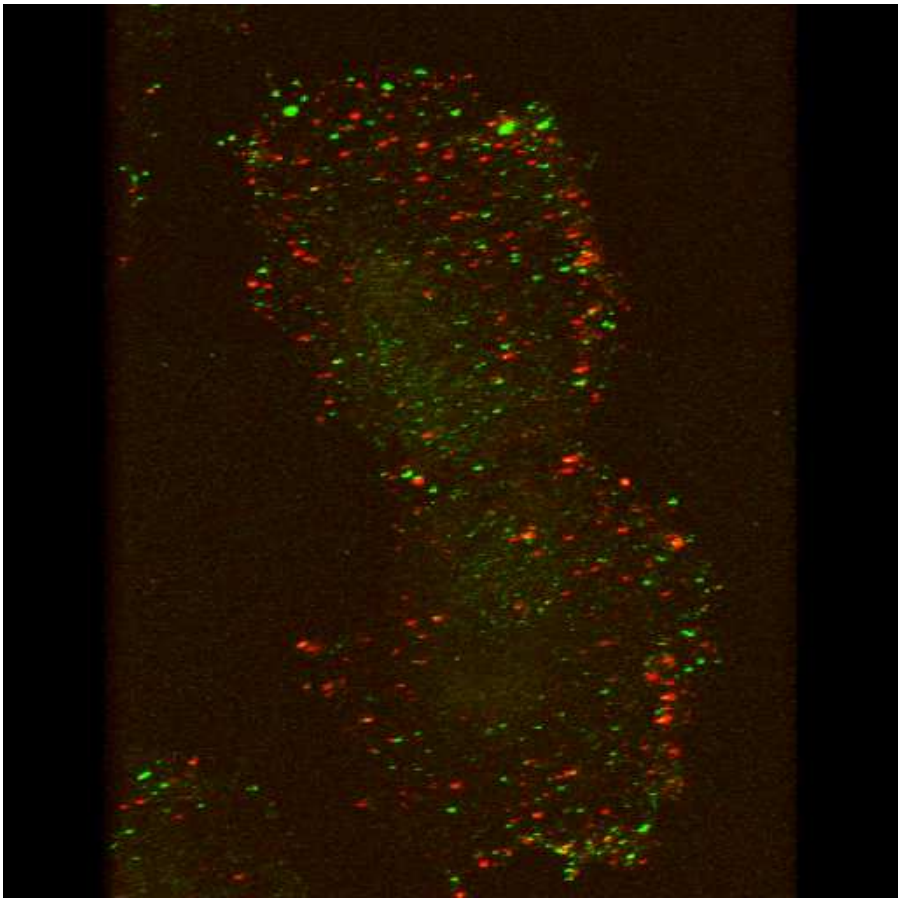

Figure 3.3: Representative confocal microscope image of TAF 15 (red) and ISG 12 (green). (n=3; each time at least 20 images were taken)

3.3 Influence of interferon α on the expression of TAF 15

Since ISG 12 is highly upregulated by type I IFNs and especially by IFNα, and since TAF 15 and TLS are also stress induced genes like ISG 12, I tested smooth muscle cells (SMC), HEK293 cells and human umbilical vein endothelial cells (HUVECS) for TAF 15 expression after IFNα stimulation.

RNAs were isolated after stimulation with 1000U/ml IFNα for 0.5 hours, 1.5 hours, 3.5 hours, 5.5 hours and 7.5 hours. Reverse transcribed cDNAs were amplified in a Roche Diagnostics LightCycler and gene expression was normalized to PBGD.

No significant up or downregulation of TAF 15 with IFNα was detected in tested cells. Variations in the expression levels of TAF 15 at the different time points may be the result of other IFNα induced mechanisms in the cell. But TAF 15 itself does not seem to be induced in an IFNα dependent manner.

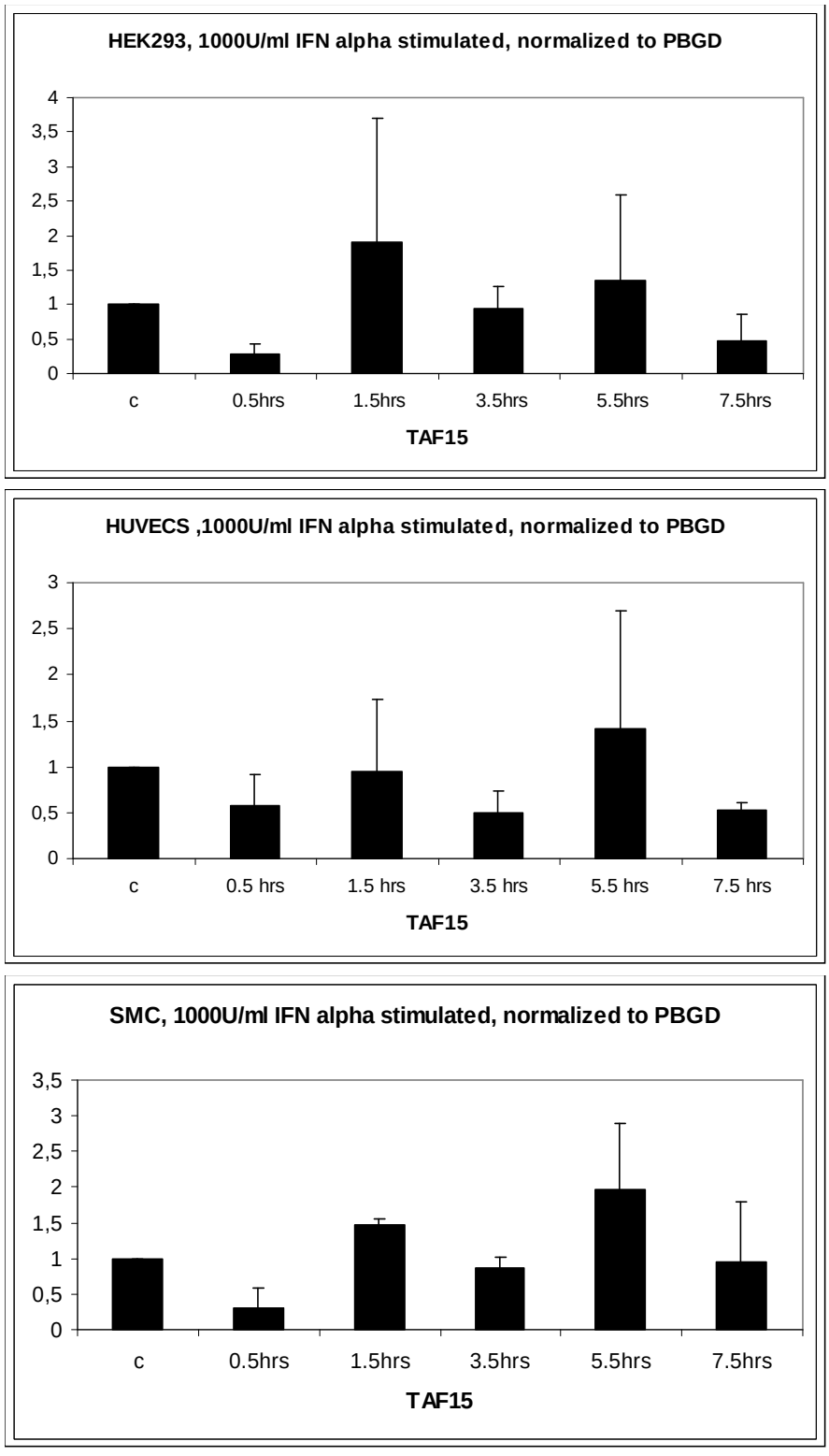

Figure 3.4: Time course for the expression of TAF 15 after stimulation with 1000U/ml IFNα in HEK293, HUVECs and SMCs. (n=3)

3.4 Influence of TAF 15 on the transcriptional activity of NR4A1

As already mentioned before, ISG 12 has the ability to almost completely abolish the transcriptional activity of NR4A1. To see whether TAF 15, as a newly identified ISG 12 partner, has an effect on the transcriptional activity of NR4A1, I performed a reportergene assay overexpressing NR4A1 and silencing TAF 15 with its mRNA specific siRNA. Scrambled RNA was used as a control. The promoter of the Luciferase reporter gene contained four NBREs and SV40 renilla was used as an internal standard.

Custom designed siTAF 15 was previously tested in HEK293 cells and real-time PCR results proof that TAF 15 is actually downregulated by this siRNA by 80%.

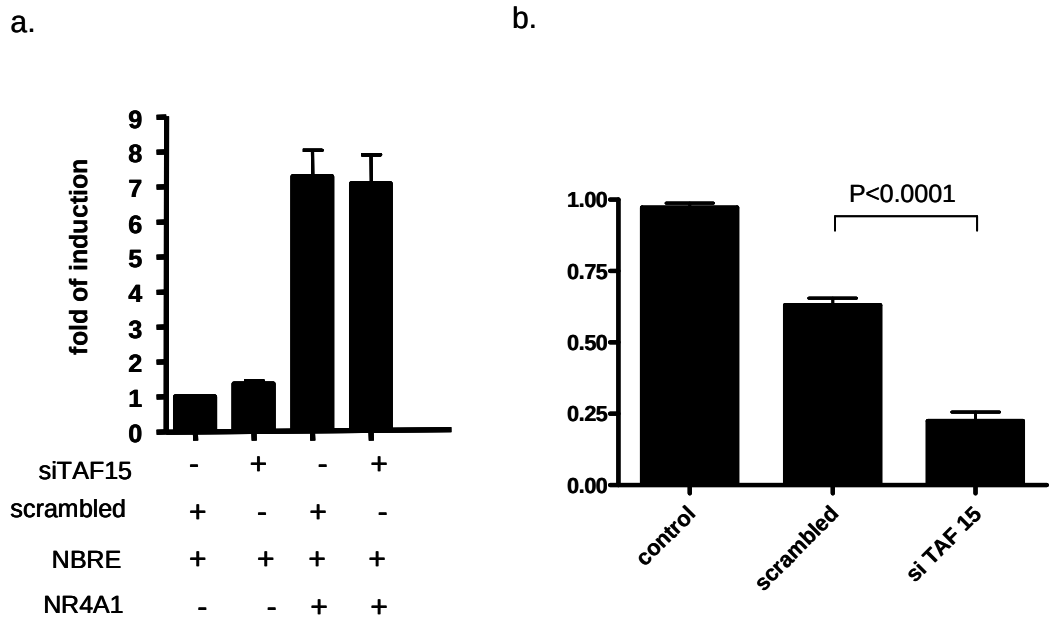

Figure 3.5: (a) Reportergene Assay for the transcriptional activity of NR4A1 using TAF 15 siRNA and scrambled RNA. (b) Real-time PCR to show the silencing effect of si TAF 15.

NR4A1 upregulates the expression of the reporter gene approximately seven fold when at the same time treated with scrambled RNA. Silencing TAF 15 does not have any effect on this upregulation, suggesting that TAF 15 itself does not influence the transcriptional activity of NR4A1.

3.5 Interactions of TAF 15 with transcription factors

We already knew that ISG 12 forms complexes with a variety of nuclear receptors, like NR4A1, RXRα and PPARα as well as other factors like p65, one part of the heterodimeric transcription factor NFκB. Furthermore, TLS, a family member of the TET family, was also already published to interact with several nuclear receptors. The possibility that ISG 12 actually forms a complex with these proteins together with TAF 15 could not be excluded. First I performed Co-Immunoprecipitations to look for interactions between TAF 15 and the previously named transcription factors, to see whether TAF 15 interacts with NRs similar as TLS. The transcription factors were overexpressed by a transient transfection of their expression vectors and used for the pull down. TAF 15 was only present at endogenous levels. TAF 15 was also detected in the cell lysate, aliquoted before the immunoprecipitation, as a positive control.

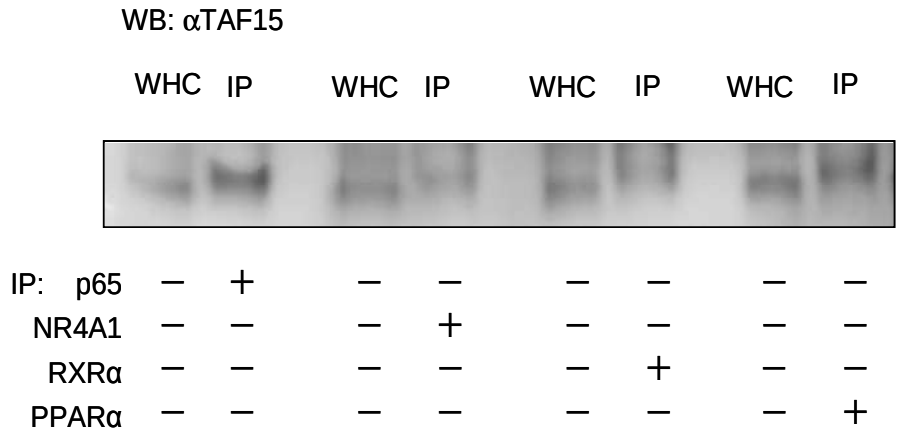

Figure 3.6: CoIPs between p65, NR4A1, RXRα, PPARα and TAF 15. The indicated transcription factors were used to pull down TAF 15. The Western blot was developed with a TAF 15 polyclonal antibody. Negative controls were performed using unspecific IgGs (data not shown). (n=4)

All four transcription factors show a positive CoIP with TAF 15, which indicates a major role of TAF 15 in the regulation of gene expression induced by these factors.

3.6 Influence of TAF 15 on complex formation between ISG 12 and transcription factors

To investigate whether TAF 15 has an influence on the complex formation between ISG 12 and certain transcription factors, I again performed several Co-Immunoprecipitations. The transcription factors were again used to pull down protein complexes, but this time I detected ISG 12 on a Western blot in either presence or absence of siTAF 15.

The detected Western blot bands were normalized to the respective ISG 12 bands from the cell lysates (not shown). The experiment was repeated three times and the quantified results were merged together to calculate statistical significance. Quantifications were done using the Imager analysis software (Alpha Innotech Corporation, USA).

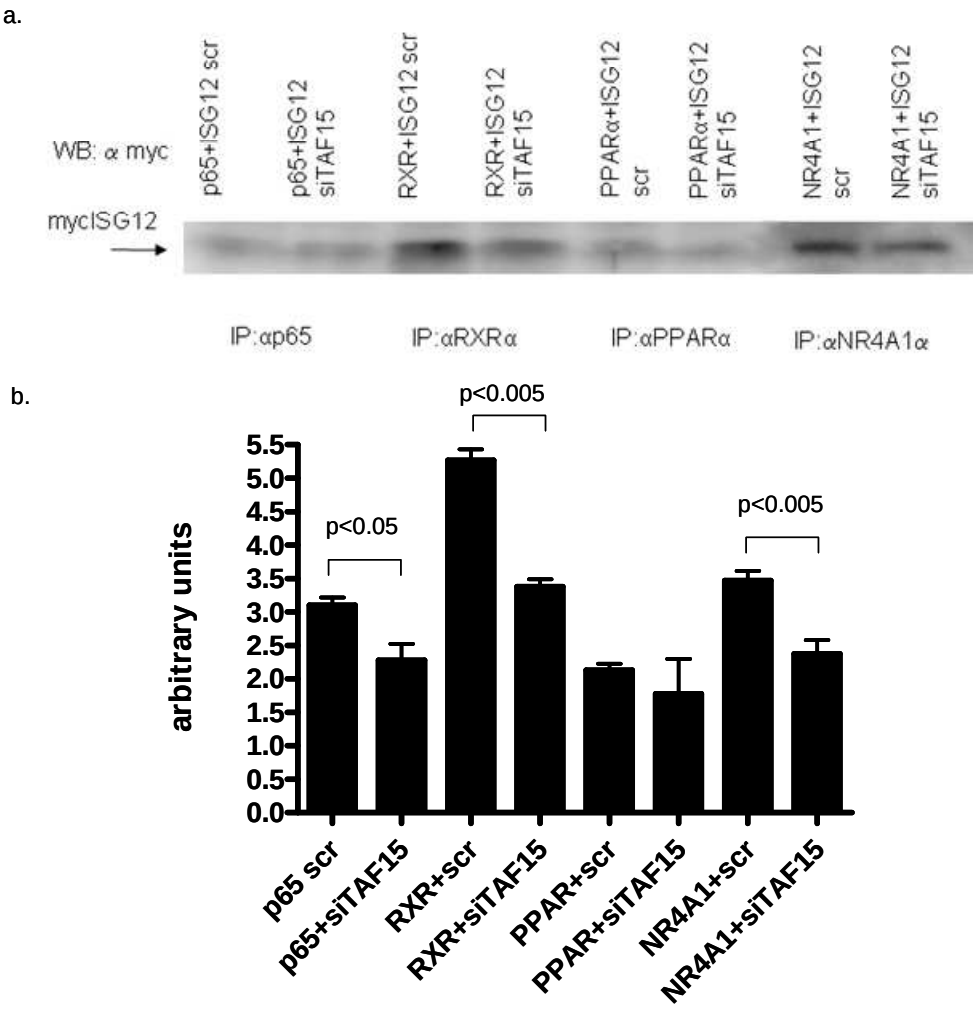

Figure 3.7: (a) Immunoreactive bands of mycISG 12, after pull-downs with P65, RXRα, PPARα and NR4A1. (b) Differences in band intensity comparing scrambled RNA and siTAF15. The immunoreactive bands were normalized to corresponding bands from cell lysates (not shown). (n=3, ttest $p<0.05$)

All but PPARα show a statistically significant reduced complex formation when TAF 15 is silenced by siRNA.

3.7 Effect of TAF 15 on the nuclear export of NR4A1

Nikolina Papac Milicevic et. al. previously showed that ISG 12 exhibits at least a part of its downregulating effect on the transcriptional activity of NR4A1 by mediating its export from the nucleus to the cytoplasm. The complete mechanism behind this effect is still not completely clarified. Since ISG 12 and TAF 15 form a complex with each other as well as with NR4A1, I looked whether the effect of ISG 12 on the translocation of NR4A1 is TAF 15 dependent or not.

I transiently transfected HEK293 cells in 2 well chamberslides with EGFP-NR4A1 and ISG 12 for immuno-histochemistry. Pictures were taken with an Olympus AX-70 microscope and cytoplasmic NR4A1 was quantified. As in previously performed experiments, siTAF 15 was used to silence endogenous TAF 15, whereas scrambled RNA was used as a negative control.

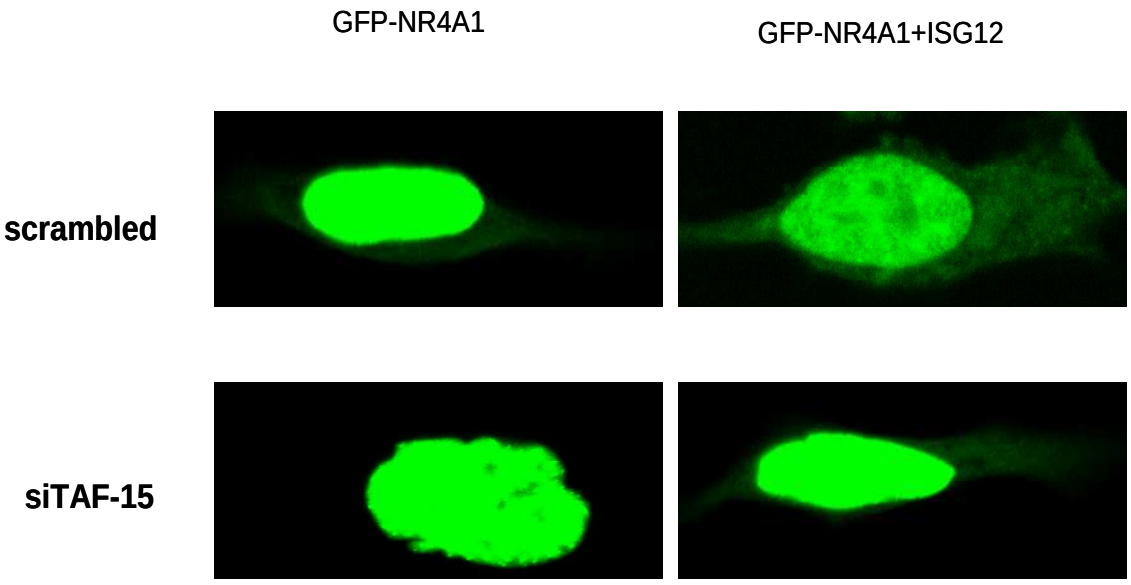

Figure 3.7: Microscopy pictures of HEK293 cells, transiently transfected with GFP-NR4A1 and ISG 12. NR4A1 is shifted into the cytoplasm when cotransfected with ISG 12, but remains in the nucleus when TAF 15 is silenced at the same time. (n=3 experiments, representative pictures)

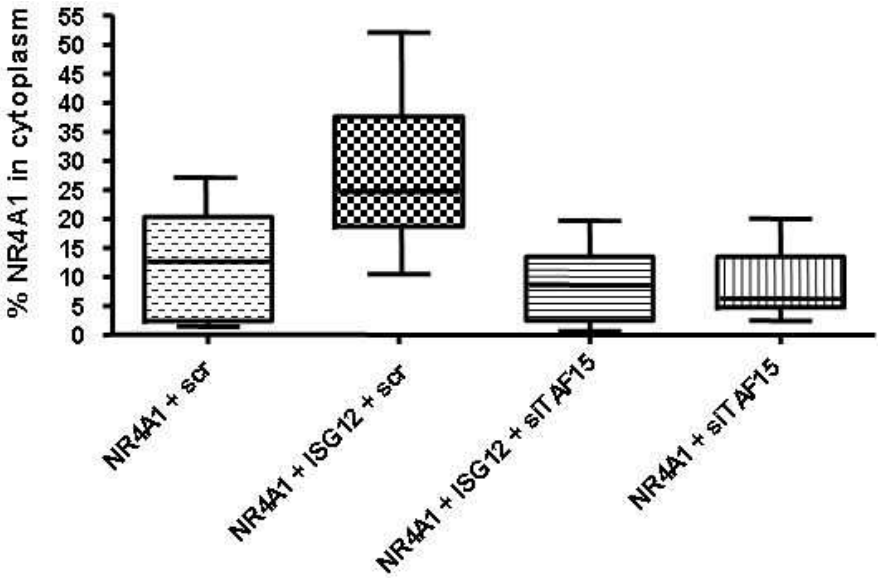

Figure 3.9: Quantification of cytoplasmic NR4A1 in transiently transfected HEK293 cells. (n=3)

Confirming the previously obtained data from *Nikolina Papac Milicevic et. al.,* approximately 40% of NR4A1 present in the cell were located in the cytoplasm when co-transfected with ISG 12. When NR4A1 is transfected alone, only approximately 20% of NR4A1 are found in the cytoplasm.

In the case of TAF 15 silencing, a co-transfection of ISG 12 doesn't change the observed outcome. In both cases, no matter whether ISG 12 is overexpressed or not, only about 15% of NR4A1 is located in the cytoplasm, indicating an involvement of TAF 15 in translocation of NR4A1. The exact mechanism of this effect is still unknown, but this experiment shows that TAF 15 is very likely involved in the exporting effect of ISG 12 on transcription factors.

3.8 FRET on NR4A1 and ISG 12 under the influence of TAF 15

To further investigate the complex formation between NR4A1 and ISG 12, I carried out a fluorescence resonance energy transfer (FRET) between them. I again used siRNA against TAF 15 to look whether TAF 15 is involved in this complex formation.

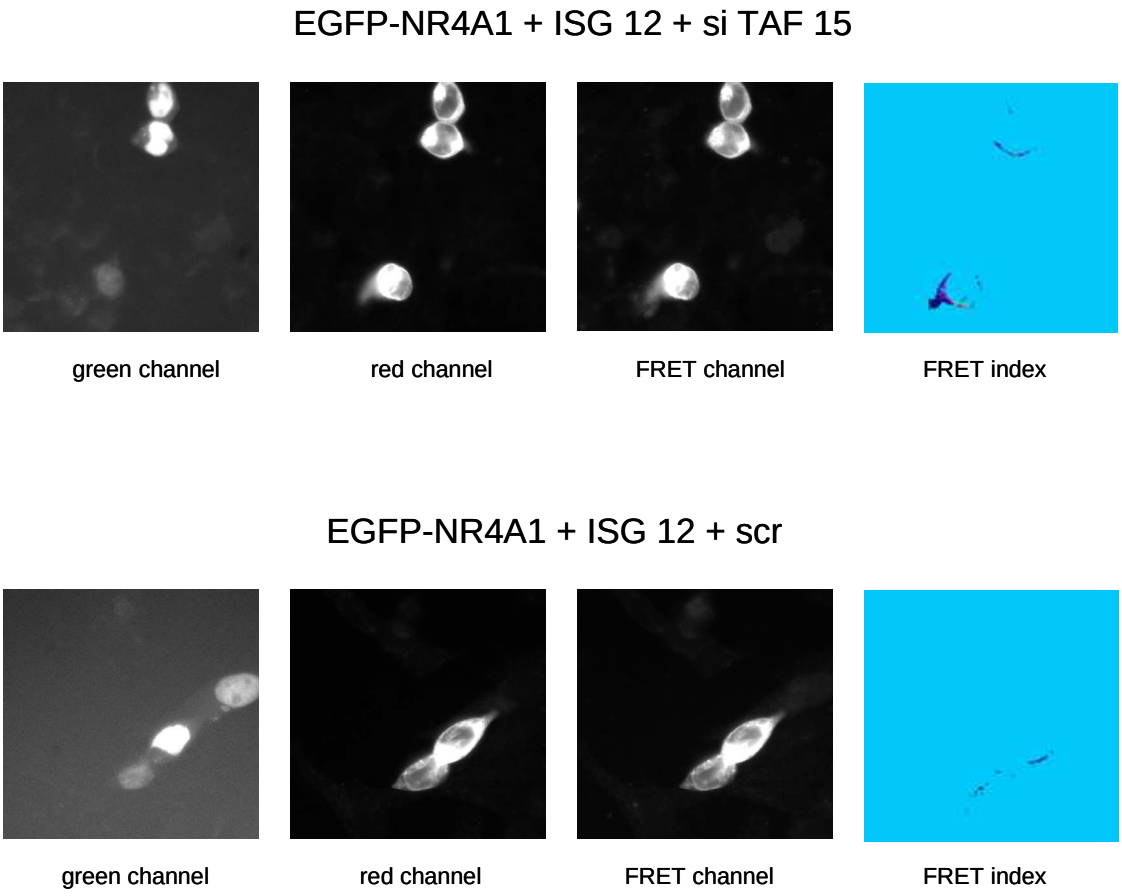

Figure 3.10: Microscopy pictures of HEK 293 cells, overexpressing EGFP tagged NR4A1 and myc tagged ISG 12. The green channel shows the EGFP-NR4A1 donor signal at an emission wavelength of 512 nm. The red channel shows the Alexa 568 labeled ISG 12 acceptor signal at an emission wavelength of 603 nm. The FRET channel shows the signal measured with the acceptor emission wavelength when excited with the donor excitation wavelength of 488 nm. The FRET index was afterwards calculated by subtracting the bleed through of both the donor and the acceptor channel.

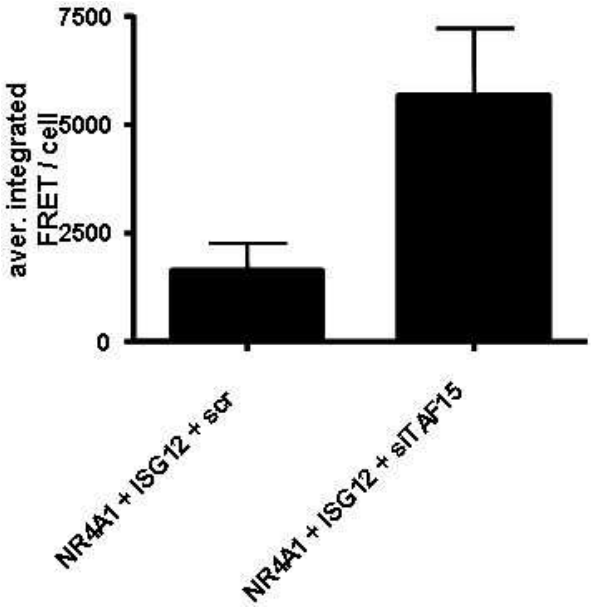

Figure 3.11: Quantification of the FRET signal between overexpressed GFP-NR4A1 and ISG-myc, treated with either scrambled RNA or siTAF 15. (n=3)

In this case, silencing of TAF 15 led to an increased interaction of NR4A1 and ISG 12. Whereas silencing of TAF 15 in Co-Immunoprecipitation experiments led to a diminished interaction. Since the interaction in CoIP experiments was not completely abolished (band still present, but with reduced intensity), it suggests that ISG 12 and NR4A1 can interact partially in a TAF 15 independent way. On the other hand, microscopy results show that TAF 15 is definitely necessary to mediate the ISG 12 dependent export of NR4A1 from the nucleus to the cytoplasm. This could mean that TAF 15 stabilizes the NR4A1-ISG 12 complex.

This is also a possible way to explain the increased FRET signal when TAF 15 is silenced. The lack of TAF 15 probably changes the way in which ISG 12 binds to NR4A1, possibly bringing the Donor and the Acceptor, necessary for the resonance transfer, closer together and thus increasing the signal.

4. Conclusion

ISG 12 is a small interferon induced protein, located in the nuclear membrane. It was found previously to be responsible for the downregulation of the transcriptional activity of several nuclear receptors, by mediating their export into the cytoplasm. To better understand the mechanism of this effect I was looking for new interaction partners of ISG 12 that might help us to better understand its role in the cellular machinery.

After evaluating possible interaction partners of ISG 12, obtained from the Tandem Affinity Purification System, TAF 15 and TLS were the first studied in the ISG 12 dependent regulation of transcriptional activity of nuclear receptors.

Concentrating on TAF 15, I primarily confirmed the rightness of the Tandem Affinity Purification System with Co – immunoprecipitation experiments in HEK293 cells and furthermore verified the colocalization of the two proteins with immuno - histochemistry images.

Furthermore I analyzed a possible change of expression of TAF 15 after an interferon stimulation, because IFNα is a major ISG 12 inducer, but no upregulation of TAF 15 on the mRNA level could be observed. This could be explained by the fact that TAF 15 is part of the basal transcriptional machinery and its role in the formation of the RNA polymerase II pre-initiation complexes is obligatory. In the case of a possible mechanism involving both TAF 15 and ISG 12, it is feasible that ISG 12 is the limiting factor. So upregulation of ISG 12 by interferon stimulation could enhance the complex formation with TAF 15 since it is already present in sufficient quantity.

ISG 12 downregulates the transcriptional activity of nuclear receptors by facilitating their export from the nucleus as already mentioned above. I did not find such an effect for TAF 15, since the transcriptional activity of NR4A1 was not affected when TAF 15 was silenced.

Both TLS and TAF 15 interact with ISG 12 and since TLS interacts with several nuclear receptors, I was interested if TAF 15 does the same. All tested NRs plus p65 were found positive to interact with TAF 15 as well as with ISG 12. The possible connection between the three components was revealed in the consequent experiment, showing significantly weaker interactions between ISG 12 and the different transcription factors when TAF 15 was silenced. Furthermore the export of

NR4A1 influenced by ISG 12 was found to be TAF 15 dependent, suggesting that TAF 15 is necessary for ISG 12 to mediate its effect.

On the other side, FRET revealed that silencing of TAF 15 led to an increased complex formation of NR4A1 and ISG 12. The possible mechanism suggested by these results is that TAF 15 may stabilize the interaction between NR4A1 and ISG 12 and by doing so helps ISG 12 to induce the export, but is not itself implicitly necessary for the formation of the NR4A1-ISG 12 complexes.

Since ISG 12 deficient mice were found to survive better in carotid artery ligation models, contrary to NR4A1 knock out mice, which showed a reduced life expectancy, it would be very interesting to fully understand the physiological interaction between these two proteins. With the performed experiments I can show that TAF 15 is definitely involved in this interplay, but the exact mechanism is yet to be resolved.

Outlook:

To further study the involvement of TAF 15 in the regulation of nuclear receptors, I would like to clone TAF 15 into an expression vector for use in reporter assays and protein microarrays. A TAF 15 deficient cell line could make the results on complex formation more significant, since siRNA never leads to a complete knock out of TAF 15.

To get more details on the complex formation involving TAF 15, ISG 12 and nuclear receptors it would be very helpful to conduct an IP over an IP, but so far I was not able to do this.

Furthermore I am interested to see if TAF 15 also influences the export of other nuclear receptors like it does with NR4A1. Finally I would like to analyze if the other TET family members exhibit the same effects as TAF 15.

5. Methods

5.1 Cell Culture

Human Embryonic Kidney cells (HEK293) were obtained form ATCC (Manassas, VA) and cultured in 75cm^2 flasks at 37℃ with 5% CO_2 in DMEM (Dulbecco's modified Eagle's medium (Sigma, St.Louis, MO)) plus 1% L-glutamine, antibiotics and 10% foetal bovine serum.

To split the cells, media was removed and 10ml of calcium-magnesium free Hank's medium (CMFH) were added. Afterwards the cells were detached with 5 ml of trypsin. After 5 minutes trypsinization was stopped by the addition of 20ml of Hank's balanced salt solution (HBSS). Cells were pelleted down and resuspended in 10ml of DMEM. The cellsupension was then added in the appropriate amount to either 6 well plates, 12 well plates or chamberslides for further experiments and to another 75 cm^2 flask for further cultivation.

5.2 Transient transfection of HEK293 with Calcium-Phosphate

HEK293 cells were transiently transfected with calcium-phosphate for Co–Immunoprecipitations, Reporter Assays and Immuno Histochemistry experiments. 1.5µg DNA per well were used in 12 Well plates and 2 well chamberslides and 3µg DNA per well in 6 well plates. DNA was mixed with dH_2O (distilled and deionized) and $CaCl_2$ and added drop-wise to the same amount of HeBS Buffer. The transfection mix was then incubated for 3 minutes at room temperature. Afterwards the DNA-precipitate was added to the cells. Transfected cells were at least incubated for 24 hours.

5.3 Dual – Luciferase Reporter Assay

5.3.1 Principle

Dual Reporter Assays are a common tool to study eukaryotic gene expression. They are especially useful to study promoter activity and the influence of transcription factors.
In this system two different reporter enzymes are used at the same time. First the activity of Firefly Luciferase is measured. The emitted light is based on the enzymes activity to decarboxylize luciferin.

$$\text{Luciferin} + \text{ATP} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{PPi} + CO_2 + \text{light}$$

The second part of the assay is the measurement of Renilla Luciferase as a standard. To be able to measure Renilla Luciferase, the Firefly Luciferase reaction is terminated by Stop & Glo.

$$\text{Coelenterazine} + O_2 \rightarrow \text{Coelenteramide} + CO_2 + \text{light}$$

As the amount of luminescence produced by Firefly Luciferase is directly proportional to the expression of our target promoter, the activity of Renilla Luciferase is not. So the activity of our target promoter is normalized to the internal control (Renilla) to minimize experimental variability due to differences in transfection efficiency and cell viability.

5.3.2 Reporter Assay

HEK293 cells were grown in 12 wells to approximately 60 – 70% confluence and then transfected with Firefly and Renilla Luciferases using the Calcium-Phosphate Method. After 24 hours of incubation the cells were lysed using 1x Passive Lysis Buffer (Promega, Wisconsin) for 30 minutes at room temperature. 20µl lysate was transferred from each well to a luminometer plate.

The Luciferase Assay Reagent and the Stop & Glo Reagent (Promega, Wisconsin) were prepared according to the manufacturer's instructions, 50µl per sample were used.

The luminescence was then measured by a Wallac Victor luminometry machine (Perkin Elmer, MA).

All experiments were performed in triplicates and repeated at least 3 times.

5.4 Co – Immunoprecipitation

5.4.1 Principle

The Co – Immunoprecipitation is a method used to find protein – protein interactions. One antibody, specific for a certain protein is used to bind its target protein, and all other proteins that are attached to it are precipitated together as a complex, bound to sepharose beads. Those beads are used to pellet down the protein complex. After dissociation from the beads proteins from that complex with the target protein can be detected in a Western blot.

5.4.2 Protocol

HEK293 cells were transfected in 6 Wells using the Calcium-Phosphate Method. After 24 hours incubation the cells were lysed for 30 minutes in a buffer containing 2.7mM KCl, 1.5mM KH_2PO_4, 9.2mM $Na_2HPO_4.2H_2O$, 150mM NaCl, 0.7% NP40, 0.3% Triton X-100 and protease inhibitor (Roche Diagnostics, Switzerland). Samples were centrifuged for 15 minutes at 15.000 x g and the supernatant was incubated for 1 hour at 4℃ with 2 µg of the appropriate antibody. The precipitation was also performed with unspecific IgGs, as a negative control. Protein A sepharose beads (Amersham Biosciences, UK) were added and the samples incubated for 2.5 hours at 4℃. Beads were washed 5 times with PBS and then resupended in 2x Lämmli-Buffer. After 10 minutes at 95℃ the supernatant was transferred into new tubes and the beads were discarded. Samples were finally subjected to Western Blot.

5.5 Western Blot

After the Co - immunoprecipitation the samples were subjected to 10% and 12% SDS-polyacrylamide gel electrophoresis. Gels where then transferred to a polyvinylidene difluoride membrane (PVDF) (Millipore, Bedford, MA). Membranes were than blocked for 1 hour using either 5% milk or 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) containing 0.1% Tween 20. The corresponding primary antibody was added directly to the blocking solution for over night incubation, in a concentration according to the manufacturer's recommendation. Afterwards membranes were incubated for 1 hour with the corresponding secondary peroxidase labeled antibodies (Amersham Biosciences, UK). ECL Plus (Amersham Biosciences, UK) was used to detect immunoreactive bands.

5.6 TAP – Tandem Affinity Purification

5.6.1 Principle

The Tandem Affinity Purification System is a well established tool to purify protein complexes under native conditions. The TAP Method requires the attachment of the TAP tag, to either the C-Terminus or the N-Terminus of the protein of interest, in our case myc-ISG 12. After stably transfecting HEK293 cells with the TAP tagged myc-ISG 12, those cells are cultivated and harvested in huge quantities. The cell extracts containing the fusion protein as well as associated partners are recovered by two specific affinity purification steps. Gathered protein complexes can then be analyzed by mass spectrometry.

The TAP tag consists of two IgG binding units of protein G from Streptococcus sp. and the streptavidin – binding peptide, separated by a TEV – protease cleavage site.

First protein complexes are pulled down by IgG sepharose and then released by TEV – protease cleavage. Cleaved proteins are furthermore bound to streptavidin sepharose and in the end eluted with biotin.

5.6.2 Protocol

5.6.2.1 Vector generation (Gateway Cloning)

The first step in generating the expression vector for TAP tagged myc-ISG 12 was to amplify ISG12 from an already existing plasmid. The PCR reaction mix contained:

1µl	template
1µl	Forward primer
1µl	Reverse primer
5µl	10x PCR buffer
5µl	dNTPs
3.5µl	$MgCl_2$
33µl	ddH_2O
0,5µl	KOD polymerase (Novagen)

Forward primer: *5´GGGG ACA AGT TTG TAC AAA AAA GCA GGC TAG ACT GCC ATG GCA TCA ATG CAG AAG CTG 3´*
Reverse primer: *5´GGGG AC CAC TTT GTA CAA GAA AGC TGG GTT GTA GAA CCT CGC AAT GAC AGC 3´*

Following thermocycler settings were used:

	98℃	2 minutes
19x	98℃	15 seconds
	55℃	5 seconds
	72℃	30 seconds
	68℃	10 minutes

KOD polymerase added the Gateway attB1 and attB2 sequences to the 5' and the 3' end of our gene of interest. The PCR product was subsequently subjected to a 1.5% Agarose gel electrophoresis and stained with 3% ethidiumbromid.
The wanted DNA band was then cut out of the agarose gel with a scalpel under UV-light and transferred to an Eppendorf tube. DNA was isolated with the QIAEX II gelextraction kit (Qiagen, Valencia, USA). The band was weighted and 3 volumes of buffer QX1 and 20µl QIAEXII reagent were added. The following purification steps were done according to the manufacturer's protocol.

The purified DNA fragment was then introduced into the Gateway Donor vector using the BP Reaction.

1µl PCR product
1µl Donor Vector
2µl BP buffer plus BP Reaction Enzym
6µl dH2O
→ 1 hour incubation at room temperature.

The enzyme mix catalyzed the recombination and insertion of the attB sequence containing PCR product into the attP recombination sites in the Gateway Donor vector. After the insertion the new vectors were called Entry vectors.

The resulting Entry vectors containing myc-ISG 12 were transformed into competent *E. coli*. Therefore 15µl *E. coli* were mixed with 10µl of the BP Reaction and put on ice for 20 minutes. Afterwards the *E. coli* were heatshocked at 42℃ for 45 seconds and put back on ice immediately for 2 minutes. The transformed bacteria were then incubated in 500µl SOC media at 37℃ for 30 minutes.

The whole mixture was plated on LB agar plates containing 50µg/ml kanamycin and incubated at 37℃ over night.

5 colonies were picked and inoculated into 2 ml of LB medium containing kanamycin. After 24 hours of incubation at 37℃ the bacteria were harvested and a minikit was performed (Eppendorf Fast Plasmid Mini-Prep Kit) according to the manufacturer's protocol.

A restriction digestion was performed as a control to see whether the DNA fragment of interest was actually inserted into the Entry vector.

10µl plasmid
1µl BsrG1 restriction enzyme
5µl NE Buffer2
34µl dH_2O
→ 2 hours incubation at 37℃ with shaking at 400 rpm .

The restricted samples were applied to an 0.8% agarose gel electrophoresis and the presence of the insert was confirmed.

The LR Reaction was done to attach the TAP tag to the C-terminus of ISG12. Again utilizing the Gateway Cloning System the LR Reaction contained:

2µl Clonase mix plus Buffer
0,8µl prV CTAP
0,4µl Entry clone
6,8µl dH_2O
→ 1 hour incubation at room temperature

The Destination vector prV CTAP contained an expression promoter and the C-

terminal TAP tag. It was kindly provided by the lab of G. Superti-Furga.

The ISG12-TAP cassette was then cloned into a Moloney murine leukemia virus-based vector, which was stably transfected into HEK293 cells and successfully transfected cells were selected by FACS analysis. This was performed by Mag. Ines Kaupe at the Research Center for Molecular Medicine.

One hundred 175cm^2 flasks were grown and the harvested cells were subjected to the affinity purification steps.

5.6.2.2 Affinity purification

Frozen cells were put on ice and resuspended with lysis buffer containing 50mM Tris/HCl, pH7.5, 5% glycerol, 0.2% NP-40, 1.5mM $MgCl_2$, 25mM NaF, 100mM NaCl, 1mM Na3VO4 and protease inhibitors. Cells were then incubated on ice for 30 minutes and centrifuged for 15 minutes at 15.000 x g at 4℃. Previously washed IgG beads (Amersham Biosiences. UK) were added to the supernatant and incubated at 4℃ for 2 hours. After incubation the beads were pe lleted down and transferred into a column (0,8ml Mobicol). Columns were washed with lysis buffer and with TEV Buffer containing 10mM Tris/HCl, pH 7.5, 100mM NaCl, 0.5mM EDTA, 0.2% NP-40 without protease inhibitors.

360µl TEV buffer plus 40µl TEV protease were added to the columns and incubated for 1 hour at 16℃. Then the TEV – eluate was allow ed to drop out by gravity flow into an Eppendorf tube. Another 400µl of TEV cleavage buffer was added to the columns and emptied by applying pressure (syringe).

Streptavidin beads were washed with TEV buffer and the bead suspension was then added to new Mobicol columns. The liquid dropped out by gravity flow.

TEV – eluate was added to the streptavidin beads and incubated for 1 hour at 4℃. The eluate dropped out by gravity flow and the beads were washed with TEV buffer. Afterwards 400µl Biotin elution buffer containing a tip of biotin diluted in 5ml 10mM Tris/HCl, pH 7.5, 10mM NaCl were added to the streptavidin beads and incubated for 5 minutes at 16℃. Again the eluate was allowed to drop out by gravity flow into an Eppendorf tube. This step was repeated once more.

The eluates were then frozen in liquid nitrogen and lyophilized over night. After that the samples were send to mass spectrometry analysis.

5.7 siRNA silencing

5.7.1 Principle

siRNA stands for small interfering RNA and is a popular mechanism to transiently silence a specific gene. It acts in the RNAi pathway by binding specifically to its mRNA target.

The RNAi pathway is usually initiated by the enzyme Dicer, which cleaves long double-stranded RNAs (dsRNA) into approximately 20 nucleotide long fragments. One of the two strands of each fragment is then incorporated into the RNA-induced silencing complex (RISC). The RISC complex then helps the siRNA to bind to its specific mRNA and induces cleavage by Argonaute, the catalytic component of the RISC complex. The cleaved mRNA is subsequently degraded and no protein product is formed, thus gene silencing occurs on a post-transcriptional level. In our case the used siRNA anti TAF15 (Ambion, Austin, USA) was already single stranded and ready to use. Corresponding scrambled RNA was used as a control.

5.7.2 PEI Transfection

5.7.2.1 Principle

PEI transfection was used to transfect HEK293 cells successfully with siRNAs. This method is based on polyethylenimine, which condenses the RNA into positivley charged particles. Nitrogens form ionic interactions with the phosphate groups on the RNA backbone. Those particles are taken up by the cell via endocytosis, the rising osmotic pressure in the endosomes causes them to burst eventually, releasing the siRNAs into the cytoplasm.

5.7.2.2 Protocol

HEK293 cells were seeded the day before in 12 Well plates and grown to approximately 50% confluence.
Before starting to prepare the transfection mixes, PEI reagent was at least vortexed for 1 minute at full speed. For one 12 well plate 554,4µl HeBS Buffer were mixed with 24µl of either siRNA (50µmol) or scrambled RNA (50µmol) in an Eppendorf tube. 554,4µl HeBS Buffer and 37µl PEI reagent were mixed separately in a second Eppendorf tube. Then the two solutions were added together and incubated at room temperature for 20 minutes. During the incubation time cells were washed twice with serum free DMEM medium. Afterwards 90µl of the transfection mix were added to 400µl of serum free DMEM and put on the cells.
After 3 hours of incubation at 37℃ the media was exchanged to 500µl pre-warmed DMEM full (containing 1% L-glutamine, antibiotics and 10 % foetal bovine serum).

On the following day cells could be transfected with plasmids of interest using the same technique. In that case 1µg DNA was transfected per well.

5.8 Immuno Histochemistry

For immuno histochemistry experiments cells were seeded in 2 well chamberslides (Labtek, Rochester, USA), which were precoated with gelatine for 15 minutes at 37℃. 1 µg DNA was transfected in each well on a 2 well chamberslide, using the Calcium – Phosphate method.
After over night incubation the cells were fixed with 4% paraformaldehyd (PFA) for 25 minutes at room temperature. PFA was removed and the cells were washed twice with 1x PBS. Permeabilization was done using 0.3% Triton X–100 in 1x PBS for 20 minutes at room temperature. Washing was repeated and the cells were blocked in 1% BSA in 1x PBS for 1 hour at room temperature.
The primary antibody was added directly into the blocking solution and incubated over night at 4℃. After washing the secondary antibody was added again in 1% BSA in 1x PBS and incubated for 1 hour at room temperature. Secondary antibodies bind to the constant Fc-portion of the primary antibodies and are labeled

with certain fluorophores, which make them detectable in a fluorescence microscope.

Then the cells were stained for 5 minutes with DAPI and afterwards the slides were mounted with coverslips using geltol.

To confirm specificity of antibodies, additional coverslides were used, stained without primary antibodies (data not shown).

Thereafter images were taken on an Olympus AX-70 microscope.

5.9 FRET

5.9.1 Principle

Fluorescence resonance energy transfer (FRET) is a physical process by which energy is transferred nonradiatively from one excited fluorophore (the donor) to another fluorophore (the acceptor). This process is distance-dependent and happens by means of intermolecular long-range dipole-dipole coupling. The combination of a cyan fluorescent protein (CFP) and a yellow fluorescent protein (YFP) is a very commonly used technique. In this case, excitation of the donor molecule (CFP) results in emission from the acceptor molecule (YFP). This mechanism is dependent on the distance between the two. Only if they are close enough (5-10nm) energy transfer will occur. Therefore FRET is a very useful method to monitor direct protein-protein interactions in living or fixed cells.

Figure 5.1: Schematic drawing of a fluorescence resonance energy transfer

5.9.2 Protocol

HEK cells were transfected and treated like for other immunohistochemistry experiments. To be able to measure a FRET signal, pEGFP-NR4A1 and ISG12-myc were transfected. ISG12-myc was then labeled with Alexa 568 fluorescent antibody in a 1:200 dilution (Molecular Probes, Oregon).
Images were then taken on a LSM-510 Meta confocal laser microscope (Zeiss, Germany) at 488 nm excitation and 512 nm emission wave length (505 – 530 nm filter for NR4A1) and separated from the Alexa 568 fluorescent label at 543 nm excitation and 603 nm emission wave length (585 – 615 nm filter for ISG12-myc).

5.10 mRNA quantification

5.10.1 RNA isolation

HEK293 cells, SMC and HUVECs were grown in 6 Well plates and stimulated with 1.000U/ml IFNα. After the stimulation the cells were harvested by removing the medium and application of 500µl QUIAzol to each well. The cells were then incubated for 5 minutes and subsequently transferred into 1.5ml Eppendorf tubes. 200µl of RNAse free chloroform were added and the tubes were incubated for 3 minutes at room temperature after vortexing. Afterwards the tubes were centrifuged at 4℃ for 15 minutes at full speed. After the centrifugation the aqueous phase of each sample was transferred into a new tube and mixed with 500µl RNAse free isopropanol. The samples were again incubated at room temperature for 10 minutes. To precipitate the RNA the samples were centrifuged at 4℃ for another 10 minutes at full speed. The supernatants were removed and discarded. The pellets were washed with 1ml of RNAse free 75% ethanol and centrifuged for 30 minutes at 4℃ at full speed. The supernatant was again discarded and the pellets were put at 50℃ in a heating block to dry. Afterwards the samples were diluted with 50µl of RNAse free water and again incubated at 50℃ for 10 minutes.

5.10.2 Reverse Transcription

The concentration of the isolated RNA was measured and adjusted to a final concentration of 3µg/µl with RNAse free water. 900ng RNA were then used to run the reverse transcription PCR, using following reaction mix:

3 µl	RNA template
1 µl	Oligo d(T)16
2 µl	10x PCR Buffer II
4 µl	25mM $MgCl_2$
8 µl	dNTPs
1 µl	RNAse Inhibitor
1 µl	MuLV Reverse Transcriptase

Following standard thermocycler program was used:

20℃	10 minutes
42℃	15 minutes
99℃	5 minutes

5.10.3 Real-time PCR

5.10.3.1 Principle

Real-time PCR is generally used to amplify and at the same time quantify cDNAs that were previously reverse transcribed from isolated mRNAs. Real-time PCR is a very useful tool to study gene expression on the level of mRNA.

In the case of real-time PCRs the DNA concentration is actually measured in real time. This is made possible by a dsDNA fluorescent dye like SYBR green. The dye only fluorescents when bound to dsDNA, and its fluorescence is measured after each cycle. The measured values of the target gene are then normalized to housekeeping genes. Housekeeping genes are believed to be equally expressed in various different tissues and are therefore suitable to be used as references. Commonly used housekeeping genes are glyceraldehyde-3-phosphate dehydrogenase (GAPDH), *β*-2-microglobulin (*β*2M), porphobilinogen deaminase (PBGD), albumin, actins or tubulins.

At some point the measured fluorescence exceeds a threshold value of background fluorescence. This point is called the crossing point and it appears as a steep rise of the curve on the experiments fluorescence chart. With a higher starting cDNA

concentration the crossing point is reached after less cycles and vice versa. That means that the number of cycles to reach the crossing point can be used to calculate the relative starting amount of cDNA used.

5.10.3.2 Protocol

cDNA was used from the previously performed reverse transcription of isolated mRNA. The final concentration of cDNA corresponded to an amount of 2,5ng total RNA. Each sample was loaded into a capillary together with 1.5 µl DNA Master Mix, 1.8 µl 25mM MgCl2, 10.1 µl H2O and 0.4 µl of each primer (10 µM). PBGD was used as a housekeeping gene. Following standard PCR program was used:

1x	10 minutes	95℃
55x	5 seconds	95℃
	15 seconds	65℃
	15 seconds	72℃

TAF 15 forward primer: *5' TGC AAT GAG CCT AGA CCA GA 3'*
TAF 15 reverse primer: *5' CCC ACT TCT ATC TCC GCT GT 3'*
PBGD forward primer: *5' TCG AGT TCA GTG CCA TCA TC 3'*
PBGD reverse primer: *5' CAG GTA CAG TTG CCC ATC CT 3'*

5.11 Statistics

Experimental values are expressed as mean ± SEM if not otherwise indicated. Statistical significances were determined by unpaired Student's t-test. Significance was assigned to a value of $p<0.05$.

6. Materials

6.1 Reagents

si TAF 15	Ambion, Austin, USA
scr RNA	Ambion, Austin, USA
ECL Plus Western Blotting Detection	Amersham Biosciences, Buckinghamshire, UK
Human Embryonic Kidney 293 cells	ATCC, Manassas, USA
Triton X-100	Bartelt, Graz, Austria
L-Glutamine	BioWhittaker Europe, Essen, Germany
PSF	BioWhittaker Europe, Essen, Germany
Trypsin	BioWhittaker Europe, Essen, Germany
Seakem GTGR agarose	Biozym, Oldendorf, Germany
Fast Plasmid Mini Kit	Eppendorf, Hamburg, Germany
Gene Ruler 1 kbp DNA ladder	Fermentas, Hanover, USA
Gene Ruler 100 bp DNA ladder	Fermentas, Hanover, USA
Ethidium bromide	Gibco, Carlsbad, USA
Alexa Fluor 488	Invitrogen, Carlsbad, USA
Alexa Fluor 568	Invitrogen, Carlsbad, USA
BP buffer plus BP reaction enzyme	Invitrogen, Carlsbad, USA
LB broth	Invitrogen, Carlsbad, USA
Protein A Sepharose beads	Invitrogen, Carlsbad, USA
Protein G Sepharose beads	Invitrogen, Carlsbad, USA
Chloroform	Merck, Darmstadt, Germany
Ethanol	Merck, Darmstadt, Germany
KOD Polymerase	Merck, Darmstadt, Germany
Protease Inhibitor	Merck, Darmstadt, Germany
PVDF Membrane Immobilon-P	Millipore Corp., Billerica, USA
BsrG1 restriction enzyme	New England BioLabs, Frankfurt, Germany
NE buffer2	New England BioLabs, Frankfurt, Germany
Streptavidin beads	New England BioLabs, Frankfurt, Germany
Plasmid Midi Kit	Qiagen, Valencia, USA
QIAEX II Agarose Gel Extraction Kit	Qiagen, Valencia, USA
QIAzol Reagent	Qiagen, Valencia, USA
QX 1	Qiagen, Valencia, USA
Fetal bovine serum	Panserin

10x PCR buffer II	Perkin Elmer, Wellesley, USA
25mM MgCl2	Perkin Elmer, Wellesley, USA
dNTPs	Perkin Elmer, Wellesley, USA
MuLV Reverse Transcriptase	Perkin Elmer, Wellesley, USA
Oligo d(T)16	Perkin Elmer, Wellesley, USA
RNAse Inhibitor	Perkin Elmer, Wellesley, USA
50x Stop & Glo Substrate	Promega, Madison, USA
Luciferase Assay Buffer II	Promega, Madison, USA
Luciferase Assay Substrate	Promega, Madison, USA
5x Passive Lysis Buffer	Promega, Madison, USA
Stop & Glo Buffer	Promega, Madison, USA
Interferon α	PromoCell, USA
10x PCR Buffer + MgCl2	Roche-Diagnostics, Basel, Switzerland
Fast Start DNA Master SYBR Green	Roche-Diagnostics, Basel, Switzerland
Light Cycler Capillaries	Roche-Diagnostics, Basel, Switzerland
MgCl2	Roche-Diagnostics, Basel, Switzerland
Agar	Sigma-Aldrich, St. Louis, USA
CMFH	Sigma-Aldrich, St. Louis, USA
DMEM	Sigma-Aldrich, St. Louis, USA
HBSS	Sigma-Aldrich, St. Louis, USA
Hepes	Sigma-Aldrich, St. Louis, USA
Isopropanol	Sigma-Aldrich, St. Louis, USA
XL 1 blue	Stratagene, Cedar Creek, USA
Smooth Muscle Cells	Technoclone, Vienna, Austria

6.2 Prepared Reagents

Ampicillin	50g/l
Biotin Elution Buffer	tip of biotin 5ml Tris/HCl 10mM, pH 7.5
Blotting Buffer	250ml 4x Running Buffer 200ml Methanol 550ml dH2O
Buffer C	6.06g Tris, pH 6.8 up to 100ml dH2O
CaCl2	29.4g CaCl2x2H2O, 2M up to 100ml dH2O filter-sterilized
CoIP Lysis Buffer	2.7mM KCl 1.5mM KH2PO4 9.2mM Na2HPO4.2H2O 150mM NaCl 0.7% NP40 0.3% Triton X-100 protease inhibitors
HeBS	8g NaCl 280mM 0.1034g Na2HPO4 1.5mM 6.5g Hepes 50mM, pH 7 up to 500ml dH2O filter-sterilized
2x Laemmli Buffer	12.5ml Buffer C 5ml β-Mercaptoethanol 2g SDS 5ml brom phenole blue
LB-Amp Agar plates	10g LB broth 7.5g Agar 50µl/l Ampicillin up to 500ml dH2O
Kanamycin	500µg/l
4% Paraformaldehyde	0.4g paraformaldehyde 10ml PBS 200µl KOH heat up to 60℃
10x PBS	400g NaCl 10g Kcl 12g KH2PO4 72g Na2HPO4.2H2O up to 5l dH2O, pH 7.4
PBST	0.1% Tween-20 in PBS

4x Running Buffer	12g Tris 57.6g Glycerol 4g SDS up to 1l dH2O
SOC Medium	0.5g LB broth 62.5µl 1M Kcl 250µl 1M MgCl2 250µl 1M MgSO4 500µl 1M glucose 25ml dH2O
Stripping Buffer	12.5ml Buffer C 5ml 20% SDS 350µl β-mercaptoethanol up to 50ml dH2O
TAE Buffer	40mM Tris-acetate 10mM EDTA
TAP Lysis Buffer	50mM Tris/HCl, pH 7.5 5% glycerol 0.2% NP40 1.5mM MgCl2 25mM NaF 100mM NaCl 1mM Na3Vo4 protease inhibitor
TEV Buffer	10mM Tris/HCl, ph 7.5 100mM NaCl 0.5mM EDTA 0.2% NP40

6.3 Machines

8. Alpha Innotech (San Leandro, CA, USA) FluorChem HD2 Imaging System
9. Amersham Biosciences (Buckinghamshire, UK) Ultrospec 3100pro Photometer
10. Bandelin (Berlin, Germany) Sonopuls UW2070
11. Bio-Rad Laboratories (Hercules, CA, USA) Power supply 1000 / 500
12. Eppendorf (Hamburg, Germany) Mastercycler ep gradient
13. Eppendorf (Hamburg, Germany) Mastercycler ep gradient S
14. Eppendorf (Hamburg, Germany) Centrifuge 5415D
15. Eppendorf (Hamburg, Germany) Centrifuge 5417C
16. Genomatix Software GmbH (Munich, Germany) MatInspector© 7.4.8
17. Hitachi Europe GmbH (Du□sseldorf, Germany) U-2000 Spectrophotometer
18. MWG Biotech (Ebersberg, Germany) UV-transilluminator
19. Olympus (Hamburg, Germany) AX – 70 microscope
20. Pharmacia (Uppsala, Sweden) Biotech Electrophoresis chamber
21. Roche Diagnostics (Basel, Switzerland) LightCycler
22. Sorvall (Kendro, Newtown, CT, USA) Centrifuge RC 26 Plus
23. Wallac (Perkin Elmer, Wellesley, MA, USA) Victor2 1420 Multilabel Counter
24. Zeiss (Germany) LSM – 510 Meta confocal microscope

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